Studies on human papillomavirus and molecular markers in head-neck cancer

Cecilia Nordfors

Stockholm 2015
Min gosse vakta dig för 3 ting;
för gamla horor
för rött vin
för nya doktorer

- Urban Hjärne
ABSTRACT

**Background.** Oropharyngeal squamous cell carcinoma (OSCC), where tonsillar and base of tongue cancer (TSCC and BOTSCC) dominate, is associated with smoking and alcohol as well as human papillomavirus (HPV) infection. The incidence of TSCC and BOTSCC, has increased lately, mainly due to HPV infection. In addition, patients with HPV-positive TSCC and BOTSCC have a better clinical outcome compared to those with the corresponding HPV-negative tumors (80% vs. 40% 5 year disease free survival (DFS)). Recently, head and neck cancer treatment has been intensified with chemotherapy and more intensive radiotherapy. This is likely unnecessary for 80% of HPV-positive TSCC and BOTSCC patients.

**Aims.** Due to the increase in TSCC and BOTSCC, we wanted to follow oral HPV-prevalence in healthy sexually active youth as well as in patients with TSCC and BOTSCC. This in order to disclose e.g. whether there were specific traits of oral HPV infection in the latter group. In addition, the presence of different HPV16 E6 variants in TSCC were analyzed as well as new biomarkers, which could aid in the identification of patients with HPV positive TSCC and BOTSCC that could be eligible for de-escalated treatment.

**Result.** In Paper I we showed that oral HPV prevalence was 9.3% among youth attending a youth clinic in Stockholm. Oral HPV infection was more common in women with genital infection and there was also HPV-type concordance between the oral and cervical sites. When testing samples from patients with suspected HNSCC in Paper II, nearly all HPVDNA+ oral samples were derived from patients with HPVDNA+ TSCC and BOTSCC. For healthy subjects with oral HPVDNA+ infection, the relative viral load was very low. In Paper III we found that the HPV16E6 L83V variant was common in TSCC, cervical cancer (CC) and cervical samples (CS), while the rare HPV16E6 R10G variant was present in a proportion of TSCC, but absent in CC and only sporadic in CS samples. Neither L83V nor R10G had any significant impact on clinical outcome. In paper IV, high number of CD8+ tumor infiltrating lymphocytes (TILs) was correlated to a better clinical outcome, especially for patients with HPVDNA+ and HPVDNA+/p16 positive TSCC and BOTSCC. CD4+ TIL counts were not linked to clinical outcome or survival for patients with HPVDNA+ tumors, although there was a tendency of better survival for patients with HPVDNA+ and HPVDNA-/p16-negative tumors. Finally in Paper V, patients with HPVDNA+ TSCC and BOTSCC and absent/weak as compared to medium/ strong CD44 intensity staining had a significantly better 3-year DFS and overall survival.

**Conclusion.** Oral HPV infection was relatively frequent in Stockholm youth as compared to other studies during the same time period, but the relative viral load was in general lower than that found for patients with HPV-positive TSCC and BOTSCC. HPV16E6 L83V variant was common in TSCC, CC and CS, while the R10G variant was present in a proportion of TSCC, but absent in CC and only sporadic in CS samples. Both high CD8+ TIL infiltration and absent/weak CD44 intensity staining seemed to be promising predictive markers for patients with HPVDNA+ TSCC and BOTSCC.
LIST OF PUBLICATIONS INCLUDED IN THE THESIS


* Contributed equally
RELATED PUBLICATIONS, NOT INCLUDED IN THE THESIS


VI. Ramqvist T, Nordfors C, Dalianis T, Ragnarsson-Olding B. DNA from human polyomaviruses, TSPyV, MWPyV, HPyV6, 7 and 9 was not detected in primary mucosal melanomas. Anticancer Res. 2014;34(2):639-43


Den här avhandlingen fokuserar på att:

1. Undersöka HPV i munhålan hos friska ungdomar för att kunna jämföra med HPV förekomst hos cancerpatienter
2. Utvärdera virala och cellulära biomarkörer som diagnosverktyg för att identifiera patienter med HPV-positiv tonsill- och tungbascancer med den bästa prognosen, för att om möjligt kunna ge dem en mindre aggressiv behandling

Fynd som gjorts är bland annat att 9,3% av friska ungdomar hade HPV i munhålan. I samma grupp ungdomar såg man också att infektioner i munhålan var vanligare bland kvinnor med en genital HPV infektion. En liknande undersökning gjordes bland patienter med misstänkt cancer i munhålan. Bland annat såg vi att patienter med tonsillcancer hade HPV i munhålan i ~80% av fallen och i allmänhet en större mängd virus i sina prover.


Därutöver genomfördes studier av cellulära markörer, i cancerceller och i omkringliggande vävnad. Arbetet fokuserade på två olika proteiner, CD8 och CD44. Den förstnämnda återfinns på vita blodkroppar som är kända för att bidra till bekämpning av bl.a. virus och bakterier. Det andra proteinet har blivit omnämnt som en bidragande faktor i andra cancerformer men deltar även i normal cellkommunikation. Studien påvisade att det är fördelaktigt för patienter med HPV att ha mycket CD8 men lite CD44.

Resultaten av dessa forskningsprojekt kan i framtiden användas för att bland annat förbättra diagnostiken av patienter med tonsill- eller tungbascancer. Ett exempel är fortsatt utveckling av vår metod för att testa HPV i munhålan hos cancerpatienter. Denna metod skulle kunna användas enkelt och smärtfritt på t.ex. en vårdcentral för att hjälpa vårdgivaren att ge ett första besked till en orolig patient. De cellulära markörerna skulle kunna användas för att bedöma hur effektiv behandlingen varit och vara till hjälp vid val av behandling. Detta skulle kunna optimera patientens välmående i förhållande till cancersjukdomen.
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<tbody>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex</td>
</tr>
<tr>
<td>ADA3</td>
<td>Transcriptional adapter 3</td>
</tr>
<tr>
<td>APL</td>
<td>Acute promyelocytic leukemia</td>
</tr>
<tr>
<td>ASCUS</td>
<td>Atypical Squamous Cells of Undetermined Significance</td>
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<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>BAK</td>
<td>Bcl-2 homologous antagonist/killer</td>
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<tr>
<td>BAX</td>
<td>Bcl-2-like protein 4</td>
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<tr>
<td>BOTSCC</td>
<td>Base of tongue squamous cell carcinoma</td>
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<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
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<tr>
<td>CC</td>
<td>Carcinoma of the uterine cervix</td>
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<tr>
<td>Cervical cancer</td>
<td>Carcinoma of the uterine cervix</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CIN</td>
<td>Cervical intraepithelial neoplasia</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CS</td>
<td>Cervical samples</td>
</tr>
<tr>
<td>DAB</td>
<td>Chromogen 3’ diaminobenzidine</td>
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<tr>
<td>DFS</td>
<td>Disease free survival</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>ddNTP</td>
<td>di deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>E2F</td>
<td>Transcription factor belonging to the E2F-family</td>
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<td>E6AP</td>
<td>E6 associated protein</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ENTKS</td>
<td>Ear nose throat clinic</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
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<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
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<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
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<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
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<tr>
<td>HR</td>
<td>High risk</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>HSPG</td>
<td>Heparin sulphate proteoglycan</td>
</tr>
<tr>
<td>HTLV-1</td>
<td>Human T-lymphotropic virus type 1</td>
</tr>
<tr>
<td>IARC</td>
<td>International agency for research on cancer</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISH</td>
<td>In-situ hybridization</td>
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<tr>
<td>KSHV</td>
<td>Kaposis sarcoma herpes virus</td>
</tr>
<tr>
<td>LCR</td>
<td>Long control region</td>
</tr>
<tr>
<td>LR</td>
<td>Low risk</td>
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<tr>
<td>MFI</td>
<td>Median fluorescent intensity</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility antigen complex</td>
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<tr>
<td>MCPyV</td>
<td>Merkel cell polyomavirus</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NCCR</td>
<td>Non-coding control region</td>
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<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
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<tr>
<td>OSCC</td>
<td>Oropharyngeal squamous cell carcinoma</td>
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<tr>
<td>p16</td>
<td>p16INK4a</td>
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<tr>
<td>p53</td>
<td>Tumor protein 53</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDZ-domain</td>
<td>PSD95-Dlg1-zo-1-domain</td>
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<tr>
<td>PML</td>
<td>Promyelocytic leukemia</td>
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<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>SAP</td>
<td>Shrimp alkaline phosphatase</td>
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<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
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<tr>
<td>STD</td>
<td>Sexually transmitted disease</td>
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<tr>
<td>TIL</td>
<td>Tumor infiltration lymphocytes</td>
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<tr>
<td>TNM</td>
<td>Tumor-node-metastasis</td>
</tr>
<tr>
<td>TSCC</td>
<td>Tonsillar squamous cell carcinoma</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>US</td>
<td>United states of America</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus like particle</td>
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1 INTRODUCTION

The principle that cancer could be transmissible between organisms was observed already in the late 19th century when the Russian veterinarian Nowinsky studied venereal tumors in dogs, which were transmissible upon subcutaneous transplantation. It was however, first in 1911, that experiments were performed demonstrating that filterable extracts from avian-sarcomas could transfer a tumor-causing agent from one individual to another. Still it was not until later that this phenomenon was proven to be caused by a virus (in this case the Rous sarcoma virus).

Many oncogenic viruses have been discovered since then, and seven of them are known to cause cancer in man (Figure 1). Two of them belong to the *Herpesviridae* family, namely the Epstein-Barr virus (EBV) and the Kaposis sarcoma herpesvirus (KSHV). EBV has a high seroprevalence in the general population (>99%) and may occasionally cause lymphomas and nasopharyngeal cancer, whereas KSHV has a much lower population seroprevalence (2% - 57%) and usually causes skin tumors and in some instances primary effusion lymphoma.

Human papillomavirus (HPV) and Merkel cell polyomavirus (MCPyV) are two other human tumor viruses causing neoplasms of the skin/mucosa. Seroprevalence for both these viruses is quite high, between 50-95%. However MCPyV is a rare cause of human cancer, while the HPV family contributes to >500.00 cases a year.

Furthermore, there are two human tumor viruses, which are known contributors to hepatocellular carcinoma, the Hepatitis B virus and the Hepatitis C virus. Even though their names imply a family relation, they do belong to different virus families (*Hepadnaviridae* and *Flaviviridae* respectively) and Hepatitis C virus has in addition been suggested to cause some lymphomas.

The human T-lymphotropic virus type 1 (HTLV-1) is another virus known to cause liquid malignancy, in this case adult T-cell leukemia, where irregularly shaped lymphocytes (leukemic cells) are observed in abundance.

![Figure 1. Timeline of the discovery of human tumor viruses. Adapted from Moore et al 2010](image-url)
The process by which a infectious agent (may it be a bacteria, virus or parasite) can cause cancer varies greatly and it is therefore helpful to divide these into two major categories; direct and indirect carcinogenesis. Indirect carcinogenesis has been described as chronic inflammation created by the agent, ultimately leading to mutations in the host cells. Direct carcinogenesis on the other hand, is induced either upon interaction between viral/bacterial proteins and the host’s cellular defense, or in the case of viruses e.g. by integration of the viral genome in the host genome itself. Examples of human tumor viruses utilizing direct carcinogenesis are HPV, MCPyV, EBV and KSHV. In the case of HPV, the virus genome may integrate into the host genome or persist in an episomal form, allowing its oncogenic proteins to be transcribed and hence maintain a transformed cell phenotype.

This thesis work will focus on the exciting world of HPV with regard to its detection in cancer, as well as in the oral cavity and the cervix in young and healthy individuals. Furthermore, immunological aspects and how one should approach cancer related to this virus will also be discussed.

1.1 HUMAN PAPILLOMAVIRUS

1.1.1 History of HPV and cancer

Harald zur Hausen was awarded the Nobel Prize in 2008 for his discovery that carcinoma of the uterine cervix (below mentioned as cervical cancer (CC)) is to a large extent caused by infection with the human papillomavirus (HPV). But the story begins much earlier than the 21st century. It was observed already in 1842 in Italy, that married women and prostitutes had a higher frequency of cervical cancer compared to nuns and virgins, which lead to a suspicion that the disease was somehow caused by sexual contact.8

In the early 1970s, this observation was attributed to herpes simplex virus type 2, but extensive studies failed to prove this hypothesis. In 1972, it was suggested and later proven, that HPV could cause cancer in epidermadysplasia verruciformis, a skin disease with uncontrollable growth of skin warts.13 This opened the door to further investigate HPV in other cancers, and in the late 1970’s evidence emerged which supported a causative role of HPV in cervical cancer. Finally in 1982, Harald zur Hausen and his colleges managed to sequence the genome of HPV6 obtained from biopsies of genital tumors.14 This was followed by the isolation of HPV16 DNA in cervical cancer biopsies in 1983.15

Soon after these findings, HPV was associated with several other types of cancer such as cancer of the vulva, vagina16, penis and anus17. These observations followed in the early 1980’s and were achieved mostly by analysis of viral DNA with Southern blotting. Reports of HPV in relation to head and neck cancer started to appear at about the same time as these findings were published.18 The reason why researchers also evaluated head and neck squamous cell carcinoma (HNSCC) in relation to HPV was most of all due to the histological similarities between bronchial and laryngeal papillomas/squamous cell carcinomas (SCC’s) compared to papilloma and SCC’s of the cervix.19,20
The discovery of HPV and its causative role in cervical cancer was for long followed by misbelief, similarly to the discovery of its relation to cancer of the oropharynx, which in the beginning was dismissed by many. However, when the International Agency for Research on Cancer (IARC) acknowledged HPV as a risk factor for oropharyngeal squamous cell carcinoma (OSCC) in 2007 this became more widely recognized.\textsuperscript{21}

Today, a lot of young women (and in some instances also young men) are vaccinated against HPV in order to relieve the burden of virus related cervical cancer worldwide. The result of these huge vaccination programs will not be detectable for many years to come, but it would also be desirable to see a decrease in HPV related head and neck cancer.

1.1.2 The viral particle and its genome

HPV belongs to the \textit{Papillomaviridae} family, which is characterized by non-enveloped small sized double stranded circular DNA viruses. The HPV capsid is approximately 55 nm in diameter with a genome of \~8000bp. One usually divides the genome into 3 parts: The early (E) and the late (L) coding regions, which both encode proteins, and the long control region (LCR) or non-coding control region (NCCR) (Figure 2).\textsuperscript{22, 23}

- The early coding region
  The early region includes six open reading frames (E1, E2, E4, E5, E6 and E7) and composes more than 50% of the viral genome. A reading frame for E8 can be found in HPV31.

- The late coding region
  The late region lies downstream of the early region and encodes two late proteins (L1 and L2), which together make up the viral capsid.

- The non-coding control region
  The non-coding control region does not have any protein-coding function itself, but contains both the origin of replication as well as binding sites for transcription factors, thus regulating the transcription of the different genes.

![Figure 2. Schematic illustration of the HPV genome (Illustration by: Nathalie Grün)](image-url)
1.1.3 Classification

Even though all HPVs have the same basic layout they do differ in several aspects and are divided into different genera, species, types and subtypes based on the relatedness of the L1 DNA sequence. There are five HPV-genera, which have approximately 10% sequence diversity of L1. These genera are named the Alpha-, Beta-, Gamma-, Nu- and Mu-papillomavirus (Figure 3). Further on, division into subtypes is based on 2-10% diversity for the whole genome, however very few HPV’s are divided this far. For some HPV types there are also different variants, these are different isolates of the same type with less than 2% nucleotide diversity. Finally, the term intratype-variant describes differences in e.g. HPV16 E6, which has five described intratype-variants: European (E), Asian (As), Asian-American, African-1 (Af-1) and African-2 (Af-2). Intratype variants have also been reported for HPV16E2 e.g. EURE2 and AsE2. Intratype-variants are assumed to have different molecular and biological features.

More than 150 different types of HPV have been reported until today, of which most result in asymptomatic infection. Some do on the other hand cause skin warts or in rare instances also malignant tumors, 12-18 are at present considered to be oncogenic. Depending on which cell surface they infect, one usually refers to the different types as either mucosal or cutaneous. The Alpha-papillomaviruses are known to infect mucosal surfaces such as the oral cavity and genital epithelium. Whereas the Beta-, Gamma-, Nu- and Mu- are associated with infection of cutaneous surfaces.

Furthermore, division into high risk- (HR) and low risk- (LR) types helps in defining whether infection is likely to result in malignant transformation or just a local wart. An infection with a LR HPV-type is not likely to cause cancer in the long run, however infection with a HR HPV-type might initiate a carcinogenic process.

![Figure 3. Phylogenetic tree displaying all the known genus of HPV and its different species](Doorbar 2006 © the Biochemical Society)
1.1.4 The viral proteins

HPV cleverly uses its host replication machinery in order to reproduce itself in large numbers. A more detailed illustration of the coding potential of HPV16 is presented in Figure 4. As the virus enters human keratinocytes, the viral genome will be transported to the nucleus in order for viral replication to take place. First, early proteins E1, E2 and E4-E7 will be transcribed. These proteins will interrupt normal cellular functions and will work in favor of increased transcription and genome replication. As infection proceeds, late proteins L1 and L2 will be transcribed and form the outer layer (or capsid) of the viral particle. Capsids will be loaded with replicated viral genomes, creating the complete virus ready for release from its host.24

Figure 4. Schematic illustration of the coding potential of the HPV16 genome.
Adapted from Zheng et al 200623

1.1.4.1 E1 and E2
The E1 protein is generally expressed at a low level and is only active in an efficient way upon interaction with E2. These two proteins together are considered to be the most important ones during early infection as they together with cellular DNA form a complex, which recruits proteins necessary for replication.24, 28

E2-proteins bind to a palindromic DNA motif in the viral genome (in HPV16 close to the origin of replication), where it recruits E1, which then may exert helicase activity. As E1 opens up the viral DNA, making it accessible to the cellular replication machinery, other cellular factors necessary for replication are recruited. Following this, E2 disassembles, enabling E1 to form a double hexameric ring, which mimics the cellular hexameric ring structures that normally form at the replication of origin in the host cell.28 E2 may also aid the segregation of viral DNA by anchoring replicating episomes to mitotic chromosomes of the host.29
At low levels of expression E2 can function as an activator of transcription, whereas at high levels E2 may displace transcriptional activators and thereby repress oncogene expression and e.g. the expression of E6 and E7. This dual capacity is believed to be due to differences in the affinity of E2 for its different binding sites, initially inducing its own transcription but later on, when E2 levels are high, repressing replication.\textsuperscript{24} Integration of viral DNA often disrupts E2 expression, hence favoring expression of E6 and E7, thereby promoting the oncogenic process of HPV. However HPV integration is not always observed in human cancer.\textsuperscript{22}

1.1.4.2 E4
E4 is translated from spliced E1\&E4 mRNAs, where the E1 and E4 ORF are fused. This protein is mostly synthesized in the late phase of viral replication and associates with the cytoskeleton in differentiating keratinocytes and is then assumed to take part in the collapse of the cytokeratin filament.\textsuperscript{30, 31}

As keratinocytes differentiate, they develop an insoluble matrix of covalently linked proteins which protect from mechanical injury. This protein matrix is referred to as the cornified envelope. Cytokeratin collapse induced by E4 is suggested to affect the integrity of the cornified envelope of the host cell, aiding the newly formed virions in their escape from the cell during productive infection.\textsuperscript{32} In addition to the just mentioned effects, E4 has the ability to relocate cyclinB/Cdk2 complexes from the nucleus to the cytoplasm, thereby inducing cell-cycle arrest in the G2-phase.\textsuperscript{33}

1.1.4.3 E5
HPV E5 is a transmembrane protein, localized mainly to the endoplasmatic reticulum (ER) and Golgi apparatus. E5 binds vacuolar proton-ATPase on cellular endosomes, ending up in disrupted endosomal pH. This change in pH blocks internalization of the epidermal growth factor receptor (EGFR). In turn, the blocking of internalization leads to EGFR being recycled back to the cell surface, increasing receptor activity and cellular proliferation. However, E5 is considered to be an oncoprotein with low transforming capacity.\textsuperscript{22, 24}

Furthermore, binding of E5 to vacuolar proton-ATPase has been observed to block the expression of major histocompatibility complex (MHC) class II antigens on human keratinocytes, which as mentioned previously are the normal target cells for HPV. However, keratinocytes do not usually express MHC class II molecules.

MHC class II expression has on the other hand been readily observed in keratinocytes in skin disorders with T-cell infiltration as well as in cell cultures upon IFN-\(\gamma\) stimulation. It has therefore been suggested that keratinocytes can work as antigen-presenting cells in a scenario where infiltrating T-cells produce IFN-\(\gamma\), thereby inducing MHC class II expression in order to evoke an immune response. As this scenario is speculative, there are several hypotheses as to how this process takes place. One suggestion is that E5 binds the C-subunit of vacuolar proton-ATPase, thereby decreasing its ability to acidify endocytic compartments. Normal acidification would induce maturation of MHC class II molecules, however when
acidification and hence maturation of MHC class II molecules is blocked, this will result in lower cell surface expression of MHC class II. The process behind this hypothetical scenario is still unclear but affords an intriguing explanation to MHC class II dysregulation.34

Effects on MHC class I expression induced by E5 have also been observed by two different pathways. Firstly, by the same mechanism as suggested for MHC class II, i.e. by binding of vacuolar proton-ATPase. Secondly, by the retention of MHC class I molecules in the Golgi apparatus by direct binding to the heavy chain.35

These two MHC dysregulation pathways result in reduced MHC class I and class II expression on the cell surface, which in turn lead to lower presentation or viral antigens, offering HPV two very effective ways of immune escape. However, deletion of E5 is frequent in cervical cancer, indicating that E5 is not necessary for transformation hence making the question of E5’s importance even more intriguing.

1.1.4.4 E6
One of the main functions of HR-HPV E6 is considered to be deregulation of the tumor suppressor p53 (Figure 5). This is achieved when E6 associated protein (E6AP) forms a complex with E6 and binds to p53, leading to its ubiquitination and degradation.36

Figure 5. The many ways in which E6 can affect its host cell and induce oncogenesis.22 E6 also targets ADA3, another histone acetyltransferase acetylating p53. However ADA3 is not only blocked by E6, but is also targeted for degradation by ubiquitination like p53.
Looking at E6 of HR- and LR-HPVs it has been observed that they do differ. Although they both bind to p53, only binding by HR-HPV E6 results in p53 degradation. The reason for this is still unclear, but it has been suggested that HR-HPV E6 can bind at two locations whereas LR-HPV E6 only binds to one, thereby only retaining the protein in the cytoplasm. Nevertheless, both HR- and LR-HPV E6 can bind directly to the p53 gene and block its transcription. Another way in which E6 can affect p53 is by preventing its acetylation and hence block stabilization of the p53 protein. This is achieved by binding of E6 to histone acetyltransferases p300 and CBP (CREB-binding protein), which would normally acetylate p53 and hence activate it.22

E6 also binds to proteins containing a PDZ-domain, inducing degradation of the same, thereby contributing to deregulation of cellular adhesion, proliferation and chromosomal integrity. E6 can also induce telomerase activity, affect interferon signaling by blocking of interferon regulatory factor 3 (IRF3) and mediate degradation of pro-apoptotic proteins (such as BAK, BAX), all contributing to further oncogenesis.22, 37

1.1.4.5  E7  
E7, more specifically HR-HPV E7, is best known for its capacity to bind and destabilize the tumor suppressors pRb, p107 and p130. All three are related and regulated by E2F-family members (Figure 6). Binding of E7 to pRb disrupts formation of the pRb-E2F complex, and this in turn releases E2F. This release will contribute to activation of other cellular effects such as G1/S-checkpoint dysregulation and cyclin A and E activation.38

Similarly to the binding difference of HR- and LR-HPV E6 to p53, a difference in the pRb binding domain has been observed between LR- and HR-HPV E7, increasing the binding affinity to pRB for the latter.37

In addition, E7 has the ability to block the effects CDK-inhibitors (cyclin dependent kinase-inhibitors) p21 and p27 and induce chromosomal instability.39 E7 also exhibits an immune modulating capacity. This capacity is initiated as E7 binds to the interferon regulatory factor 9 (IRF-9), which is involved in the signaling pathway of interferon-α (IFN-α), and by blocking the nuclear translocation of IRF-9, it hence indirectly blocks the antiviral function of IFN-α.40 Furthermore, it has been proposed that E7 may block activation of the IFN-β promoter by inhibiting IRF-1 signaling.41 This might be exerted as E7 can act directly on IFN-γ, suppressing its activation of IRF-1 and hence negatively affect MHC class I presentation and IFN-β activation.42
The most well-known effect of E7 is blocking of the retinoblastoma protein (pRb) which allows constitutive activation of E2F. This function among others has a great impact on many cellular processes inducing hyperproliferation and S-phase entry.

1.1.4.6 Associations between E6, E7, p53, pRb and p16 overexpression
E6 and E7 are the regulatory proteins that are the most important for the development of HPV induced cancer. These two proteins complement each other in the transformation process and are highly efficient in immortalizing human primary keratinocytes.37 As described above E6 acts on p53, while E7 targets Rb, and their effects are thus synergistic. Expression of E7 followed by inactivation of pRB induces an increase in the levels of p53. This increase is however counteracted by E6 as it abrogates p53 induced growth arrest, allowing uncontrolled cell growth.

This rescue of one protein effect by the other goes two ways. As mentioned above, binding by E7 to pRb followed by disruption of pRb-E2F result in increased expression of the cyclin dependent kinase inhibitor p16$^{INK4a}$ (p16). In the past p16 overexpression was regarded as a surrogate marker for active HPV infection, due to this activation.

Following this increase in p16, the effect of E6 can be impaired.39 This effect is however counteracted by E7, as it activates cyclin A and cyclin E directly, resulting in bypass of cell cycle checkpoints and hence continuous cell division.43

1.1.4.7 E3 and E8 – where did they go?
At present, the E3 gene has only been observed in a few papillomaviruses and has not been proven to encode any protein or serve any function.44
Most HPV types do not express the E8 protein. When present, the protein is translated from a spliced E8\textasciitilde{}E2 transcript, where the E8 ORF is linked to the splice acceptor in the E2 gene. The transcript has been described for some α-HPVs (HPV5, 1, 11, 16, 18, 31 and 33), but not for any β-HPV. The E8\textasciitilde{}E2 protein works as a negative regulator of viral replication, and downregulates gene expression by working as a repressor of the full length E2, by doing so inhibiting E2 repression of E6 and E7 and promoting oncogenesis.\textsuperscript{45, 46}

1.1.4.8 L1 and L2
L1 and L2 are the two capsid proteins, expressed by HPV in the late stages of the virus life cycle. The genes encoding these two proteins make up approximately 40% of the virus genome. Together L1 and L2 create the viral capsid within which the viral genome will be contained. Notable is that expression of the minor capsid protein L2 precedes that of the major capsid protein L1.

The way in which genome amplification and capsid protein synthesis are linked is not fully understood. This process is assumed to be regulated both at the protein level and by RNA processing. E2 is suggested to play a key role in the initiation of late transcription as it mediates expression of these genes via splice site usage, creating transcripts that end at the late polyadenylation site, generating mRNAs encoding L1 and L2. This is different from the strategy for expression of the early genes that are transcribed upon promoter activation and not by blocking of polyadenylation.\textsuperscript{47-49}

1.1.4.9 LCR
Transcription of all the viral proteins encoded in the HPV genome is regulated by elements located in the long control region (LCR), which contains binding sites for transcription factors, silencers and repressors. All of these regulatory elements are borrowed from the host cell replication machinery.\textsuperscript{50, 51}

1.1.5 Life cycle – viral entry, replication, assembly and release
The replication life cycle of HPV is best known in keratinocytes undergoing differentiation (Figure 7.). The virus infects epidermal or epithelial surfaces and replicates upon cellular differentiation. Viral gene expression then goes through different stages as the cells mature. Early genes are expressed in the lower parts of the epidermis, in the basal compartments, whereas expression of viral DNA and capsid proteins takes place in the suprabasal part. Viral assembly takes place in terminally differentiated cells.

Infection with HPV does not normally cause cancer. However, when viral oncogene transcription takes place, disturbing normal cellular activity in combination with e.g. lack of immune recognition by the host cancer development may occur.\textsuperscript{3} Moreover, upon viral integration transcription of viral oncogenes can be enhanced and functional mutations may also occur.\textsuperscript{37, 39}
1.1.5.1  **Viral entry**
In most cases HPV enters the body via a small wound in the skin or mucosa of the host, gaining direct access to its target cells. Stratified squamous epithelium is the preferred entry point for HPV, but cell junctions between different types of epithelial cells such as those in the cervix uteri are also targeted. HPV firstly bind to heparin sulfate proteoglycan (HSPG) - receptors on the epithelial cell surface or on the basement membrane. Binding to the extracellular matrix (ECM) is another way in which HPV can facilitate cellular attachment. When binding has been established, conformational changes of the capsid and proteolytic cleaving of L2 occurs followed by endocytosis. This process is suggested to be independent from clathrin-, caveolin-, lipid raft-, flotillin-, cholesterol-, and dynamin-independent.47, 52

1.1.5.2  **Intracellular transport**
As acidification of the endosome takes place, the virion is disrupted revealing an L2-viral DNA complex. This complex is transported by actin protrusions, delivering its cargo to the trans-Golgi network, where the final nuclear transport is believed to take place via microtubules. The process of nuclear entry is not yet fully understood, but recent research indicates that mitosis is necessary, since the breakdown of the nuclear envelope facilitates viral-DNA to associate with chromatin. L2 is assumed to mediate this association since it is the only molecule following the viral-DNA into the nucleus. However, this is also still unclear.

1.1.5.3  **Replication**
After entry into the nucleus the genome is stabilized as an episome, from which initial replication and transcription will occur. Transcription is initiated from the early promoter and occurs only from one strand of DNA. As mentioned previously, E2 binds to the viral genome and recruits E1, which then opens up the viral DNA for transcription. A majority of the HPV genome transcripts are polycistronic. Hence the pre-mRNAs generated will be spliced into smaller mRNA pieces encoding different viral proteins. Protein translation takes place in the cytoplasm, by the use of the protein machinery of the host cell.

During early infection, all the early viral proteins (E1, E2, and E4-E7) are expressed. These proteins enhance cell cycle progression, repress cellular defense mechanisms and block tumor suppressors in order for the virus to replicate freely. The late proteins are transcribed as the early polyadenylation site becomes blocked by E2, allowing for longer readouts of the viral genome, finishing at the late polyadenylation site and thereby generate late mRNAs.

1.1.5.4  **Assembly and release**
As transcription of the late proteins begin, E2 associates with the viral DNA and recruits L2 to the nucleus. After translocation, L2 binds to nuclear sub-compartments so-called PML bodies in an E2 dependent manner. The function of PML-bodies is not fully elucidated but it is assumed that E6 can abrogate PML induced cellular senescence an also directs its degradation.37 Notably, PML (promyelocytic leukemia-bodies are found in abundance in patients with acute promyelocytic leukemia (APL).53
Figure 7. Life cycle – viral entry, replication, assembly and release
Initially, L1 remains in the cytoplasm and forms capsomers. Only upon L2 binding and PML component displacement, will L1 relocate to the nucleus in order to form viral capsids.\textsuperscript{54} When the complete viral particle has been formed, further maturation and transport to the cell membrane will start.\textsuperscript{55} As the now finished virus reaches the host cells outer barrier, E4 will aid in disrupting the keratin networks and open up the cornified membrane.
1.1.6 HPV detection methods

Since HPV was first discovered in skin and mucosal lesions, the most common way of sampling has been to collect specimens from these sites. However, the most effective way of doing so has been a subject of debate ever since the discovery of HPV. Regardless if actual biopsy pieces or cotton swabs from the site of interest are analyzed, the quality of the sample and the information extracted from it has always been a matter of discussion.

1.1.6.1 Southern blotting
This method was for long used as a standard method for detection of HPV DNA, but has been replaced by PCR during recent years due to the higher sensitivity of the latter method. Even so, Southern blots can identify as little as 0.1 copies of viral DNA per cell and the difference between episomal and integrated DNA can easily be detected on an agarose gel. In a Southern blot, the DNA sample is extracted and cleaved into smaller pieces by restriction enzymes, which enables size separation on an agarose gel. The size separated DNA pieces are then transferred onto a nitrocellulose membrane and hybridized with isotope-labeled (HPV specific probes) which can then be detected.56

1.1.6.2 Polymerase chain reaction (PCR)
PCR is used for direct amplification of HPV DNA and is considered to be very sensitive. Primers used for amplification are often general/consensus primers detecting many different HPV types but with varying binding/detection sensitivity.

Examples of general primer pairs are GP5/GP6, MY09/MY11 and SPF primers, which all bind to the L1-region.57-59 In order to overcome the lower specificity for some HPV types due to mismatches between the primers and viral sequence, the annealing temperature during the PCR is usually lowered. The GP5/GP6 primer sequence can also be modified, such as extending them with 3nt, as in the Gp5+/Gp6+ primers.60 This system enables detection of HPV types, which previously have been hard to identify in multiplex setting. An alternative is to use a mixture of different variant Gp5/Gp6 primers as described for the bead-based multiplex assay below.61 Another example of general primers are CPI/CDIIG, amplifying the E1-region.62

After amplification, in order to verify presence of viral DNA, the most common method used previously was gel electrophoresis, where amplicons were separated depending on size. However, when general primers are used the different types HPV cannot be discriminated and for this, complementary type specific PCR or sequencing is needed.56

An alternative way to directly identify the HPV type is to use a probe based method such as InnoLiPa, which detects multiple types displaying HPV presence by color changes on a strip.63 Another, recent approach, is the use of a bead-based assay, which allows multiplex type-specific detection by the use of specific probes attached to beads (as described below in section 3.3.2).
1.1.6.3 In situ hybridization (ISH)
With ISH e.g. a biotinylated probe (alternatively also labeled with fluorochromes or radiolabels) is first hybridized to the viral DNA, where after the signal from the probe can be detected and amplified depending on the detection molecules. Presence and localization of the DNA can then be evaluated by e.g. light or fluorescence microscopy.\(^{56}\)

1.1.6.4 Detection of serum antibodies
As the immune system fights against an HPV infection, B-cells start to produce antibodies (Ab) against the capsid protein L1. This happens in ~50% of infected individuals.\(^{64,65}\) Since anti-L1 antibodies are common they are not very useful as diagnostic tool or as a sign of tumourigenesis, but can instead be regarded as a indication of a past or present HPV infection.\(^{66}\)

Ab responses to E6 and E7 are not very common. In addition, recent studies have confirmed that presence of Ab against HR-HPV E6/E7 in sera is of note. These Ab may also have diagnostic value in both cervical cancer and OSCC, especially for OSCC, where antibodies have been observed as early as 10 years before diagnosis.\(^{67,68}\) Previous studies have often focused on HPV status determination upon discovery of a tumor, but the opportunity to early on determine serological status as a risk factor in cancer formation is a relatively new approach.\(^{69}\)

1.1.6.5 Immunohistochemistry for viral proteins
IHC for viral proteins such as E6 and E7 has been performed, however with low success rate due to poor sensitivity and specificity, which has led to that this method is not used for routine testing. However, recent studies investigating the possible use of E7 staining in cervical cancer have shown promising results discriminating between high- and low-grade neoplasia. To confirm these findings, further investigations need to be performed, especially in relation to HNSCC.\(^{70}\)

1.1.6.6 Immunohistochemistry for p16
In the past overexpression of p16 was considered to be a reliable pseudo-marker for active high risk HPV infection, with functional E7 protein.\(^{71}\) However, IHC for p16 is not really a detection method for HPV. Today this method is routinely used to assess p16 expression levels, but the role of p16 overexpression has become more nuanced.

In cervical cancer, upregulation of p16 has been found to be a marker of clinical outcome, predicting risk of progression from low grade CIN to higher grades. The same clear correlation has not been observed in the case HNSCC and OSCC, although p16-positivity is very commonly observed in correlation with infection with HR-HPV.\(^{72}\)

In addition, the combination of p16 overexpression together with presence of HR-HPV DNA in e.g. OSCC is regarded as almost as sensitive as detecting HR-HPV mRNA.\(^{73}\)
1.1.7 HPV prevalence in normal population

HPV is one of the most common sexually transmitted diseases in the world. It has been estimated that ~70% of the sexually active population will be infected with HPV during their lifetime. For most of these individuals, an HPV infection will be cleared by the immune system within <2 years (with a median of approximately 10 months). However, for women with persistent infection with HR-HPV, the risk of developing cervical cancer increases drastically.

1.1.7.1 Genital HPV infection

Infection is common for both men and women, but the time point/duration of initial infection seems to differ. It has been observed that women have a peak in infection rate in their early 20’s, as many become sexually active and may have multiple partners. In some studies a second peak has been observed at the age of 45-50 years of age, the reason for this is not fully elucidated, but it is likely caused by changes in sexual behavior or maybe hormonal changes pre-menopause. In the male population, a more even distribution of infection throughout life has been noted; after an initial peak a more steady prevalence has been observed. This is assumed to derive from more frequent reinfections, as compared to females, although this is highly speculative.

To estimate the actual prevalence in healthy individuals has proven tricky and rates vary widely between different studies, sampling techniques and the sensitivity of the assay used. A meta-analysis produced in 2010, combining 194 studies performed in 59 countries, suggests a world prevalence of 7.2% (11.7% adjusted) in healthy women with normal cytology. However the country specific prevalence varied between 1.6% and 41.9% with the greatest numbers (adjusted) found in the Caribbean (35.4%), Eastern Africa (33.6%) and Eastern Europe (21.4%). In contrast, the lowest prevalence was found to be 1.7% in Western Asia and 4.7% in Northern America. In the very same study, one could observe that women with a mean age below 25 years had a much higher HPV-prevalence compared to other age groups. The prevalence then decreased with increasing age until a mean age of ≥55 years was reached, then the prevalence increased again.

This pattern of higher prevalence among younger women has been observed in other studies as well. A study performed at a youth clinic in Stockholm, Sweden, found a cervical HPV prevalence of 60-70% if including both HR and LR HPV types in young women aged 15-23 years of age. However, it is now well established that; In general cervical HPV prevalence usually decreases after 30 years of age, and continues to do so as women approach their 50’s, where the prevalence again increases slightly.

1.1.7.2 Anal HPV prevalence

There is a clear difference in anal HPV-prevalence between men and women. The highest prevalence has been observed in men who have sex with men (≥50%, depending on population), followed by women who have sex with men (~30%) and finally men who have sex with women (~12%, almost half compared to women) according to a newly published
1.1.7.3 Oral HPV prevalence

Oral HPV-prevalence has not been as well studied as compared to genital and anal infection, but some trends have however been observed. Men have higher oral-prevalence compared to women in some studies. This could be due to that females in general have both a higher genital prevalence and higher genital viral load as compared to men. Thus the latter are more likely to get infected upon oral sex than women. Data also indicate that longtime monogamous relationships protect men from both genital and oral re-infection by HPV. Other contributing factors could be that men usually have more sexual partners (hence greater exposure) and that women might have some level of systemic immunity already present due to previous cervical infection, making them more resistant to oral infection. It has been estimated that approximately 1% of healthy individuals have an oral HPV infection. However, as mentioned above, the numbers of studies on oral HPV prevalence are limited.

One aim of this thesis was therefore to investigate oral HPV prevalence in youth.

1.1.8 HPV associated tumors

Tumors harboring HPV can, depending on HPV type, be benign, e.g. skin warts, genital condyloma and recurrent laryngeal papillomas. They can also be malignant e.g. cancer of the cervix, the anogenital tract or the oropharynx. In this chapter, we focus only on malignant tumors (Table 1).

Since the 1980’s, HPV has been accepted as a contributor of cervical cancer. This made associations with other anogenital cancers easier to accept. The association of HPV to tumors in the head neck region was on the other hand doubted for a much longer time. Finally in 2007, the International Agency for Research on Cancer (IARC) acknowledged HPV16 as a risk factor for OSCC, in addition to smoking and alcohol.

HPV was originally assumed to cause laryngeal cancer, since HPV caused recurrent papillomatosis of the larynx. However, when investigating different tumor sites in the oral cavity in order to find were HPV was most common, it was observed that HPV was most frequent in OSCC, especially in tonsillar and base of tongue cancer. The most prevalent type was HPV16, representing ~90% of the cases.
Table 1. HPV associated cancer prevalence worldwide, data and estimated percentage of HPV positive tumors

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Prevalence, Worldwide</th>
<th>HPV attributable fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cervical cancer</strong></td>
<td>530.000</td>
<td>&gt;95%</td>
</tr>
<tr>
<td><strong>Head and neck cancer</strong></td>
<td>563.826</td>
<td>~25%</td>
</tr>
<tr>
<td>OSCC</td>
<td>85.000</td>
<td>12-80%</td>
</tr>
<tr>
<td><strong>Vulvar cancer</strong></td>
<td>40.000</td>
<td>~40%</td>
</tr>
<tr>
<td><strong>Vaginal cancer</strong></td>
<td></td>
<td>70%</td>
</tr>
<tr>
<td><strong>Penile cancer</strong></td>
<td>26.300</td>
<td>50%</td>
</tr>
<tr>
<td><strong>Anal cancer</strong></td>
<td>30.000</td>
<td>88-90%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>610.000</td>
<td>5% of all cancer</td>
</tr>
</tbody>
</table>

1.1.8.2 Cervical cancer and HPV
Worldwide, cervical cancer is the third most common cancer type affecting ~530.000 women every year, being responsible for about 266.000 cancer related deaths/year. The IARC classified HPV16 and HPV18 as carcinogenic for cervical cancer in 1995 and it has since then been proven that HPV is strongly associated to cervical cancer and pre-cancers of this site. As mentioned previously, most HPV infections are recognized by the immune system and heal in <2 years. Only 10-30% of the infections remain detectable for more than 1 - 2 years. However, in the case of persistent infection with a HR-HPV type (such as 16 or 18) the risk of developing cervical cancer increases drastically. More than 90% of all cervical cancer cases are related to an HPV infection and the most common types causing the disease are HPV16 and HPV18, together being responsible for ~70% of the cases. An HPV infection can cause both squamous cell carcinoma (SCC) and adenocarcinoma (ADC) even though SCC is more common.

1.1.8.3 Anal cancer and HPV
The prevalence of anal cancer has been estimated to ~1 in 100.000 in the general population (with a slightly higher incidence for women) generating about 30.000 cases a year worldwide. Out of these, 90% are assumed to be caused by HPV, with an even distribution among male and female cases. However, when it comes to studies of anal HPV-prevalence, there has been a focus on studies with male subjects, often homosexual, even though the prevalence of anal cancer among females is slightly higher.
1.1.8.4 Penile cancer and HPV
The prevalence for penile cancer is very similar to that of anal cancer with ~1 in 100,000, where the fraction contributed by HPV has been estimated to be about 50%. 83, 97

1.1.8.5 Head and neck squamous cell carcinoma (HNSCC) and HPV
Head and neck carcinoma is the sixth most common cancer in the world, representing almost 3.5% of all tumors. Ninety percent of these are head and neck squamous cell carcinoma (HNSCC), most of which arise in the oropharynx, hypopharynx, larynx or in the oral cavity. The 5-year survival rate has been estimated to <50%, providing patients with a very poor prognosis.102 The prevalence of HPV in HNSCC varies, but has been documented to be highest in OSCC.103 Furthermore, in many Western countries the incidence of HPV-positive OSCC, especially in tonsillar and base of tongue cancer (TSCC and BOTSCC) has increased.104 This has not been shown for other HNSCC, where the prevalence of HPV has been reported to be around 22% or less.105 Moreover, the numbers of HPV-positive tumors in non-oropharyngeal HNSCC have not been observed to increase.105, 106

1.2 OROPHARYNGEAL SQUAMOUS CELL CARCINOMA (OSCC)
Out of the 600,000 possibly HPV associated cancer cases diagnosed each year 10% are OSCC and more than three quarters of these are diagnosed in men.107 Risk factors, which contribute to disease progression in OSCC, include smoking, alcohol abuse and betel nut chewing (common in southeastern Asia).108, 109 In addition, the discovery and acknowledgement by the IARC in 2007 that HPV was a contributing factor to OSCC has made unprotected sex an additional risk factor.

1.2.1 Anatomy of the oropharynx
The oropharynx is located at the back of the oral cavity, in the middle part of the pharynx, and consists of four sub-sites: the palatine tonsils, the base of tongue, the soft palate and the walls of the pharynx which are all covered by squamous epithelium (Figure 8). Within this area there is a ring of lymphoepithelium often referred to as Waldeyer’s ring, which to some extent encircles the oropharynx. This ring includes the lingual tonsils, the palatine tonsils and the inferior portion of the nasopharyngeal tonsils (adenoids). The lymphatic epithelium of the oral cavity is a squamous cell epithelium, which invaginates and merges with the underlying lymphoid tissue, forming crypts, which are often found on the palatine tonsils. There can be 10-30 crypts/tonsil in the palatine tonsils, while crypts are more rarely found in the tongue base, usually only one, and lacking in the nasopharyngeal tonsils.

Due to its specific epithelial structure, tumors arising in the Waldeyer’s ring often metastasize early and in the case of very small tumors there will often be neck lymph node metastasis discovered prior to an actual find of a primary tumor.93, 110
Figure 8. The oropharynx is located in the back of the oral cavity and includes the base of tongue, tonsils, soft palate and pharyngeal walls (not displayed in the picture). 

Illustration by Nathalie Grün

1.2.2 Prevalence of HPV in OSCC

The prevalence of HNSCC has declined over the last decades in the western world, where smoking habits have decreased. However the prevalence of OSCC as a separate group has increased. This trend has been reported in many countries such as U.S., Sweden, England, Scotland, Australia, Finland, Spain, Canada, Portugal, New Zealand, the Netherlands and Denmark. Even more intriguing is that HPV-positive OSCC seems to be increasing, whereas HPV-negative OSCC has been decreasing as illustrated for TSCC in Stockholm Sweden from 1970-2007 (Figure 9). Similar data have also been shown for BOTSCC in Stockholm, Sweden and OSCC in the U.S.

Figure 9. Estimated age-standardized incidence for HPV-positive and HPV-negative tonsillar squamous cell carcinoma (TSCC), Stockholm Sweden.
The HPV prevalence in OSCC has been reported to vary over geographical regions with about 60-70% in the United States, ~40% in Europe and 46% in Asia. The reason for these variations is unknown, but they are assumed to be due to lifestyle differences. HPV prevalence may also vary between tumor sites. A meta-analysis from 2014 indicated a 45.3% HPV prevalence in tonsillar cancer compared to 39.6% in the pharynx. Notably, HPV16 dominates excessively in OSCC, whereas other types such as 18, 33, 31 and 35 are found less frequently.

1.2.3 OSCC and clinical outcome
As mentioned briefly above, HPV-positive OSCC, especially HPV-positive TSCC and BOTSCC have a better clinical outcome as compared to the corresponding HPV-negative cancers (80% vs. 40% respectively 5-year disease free survival) (Figure 10). This has been found to be the case even with conventional radiotherapy and surgery.

Figure 10. Survival rates, tonsillar cancer in Sweden

The reason for the discrepancy is not yet known between patients with HPV-positive tumors and HPV-negative tumors. It has on the other hand been shown that patients with HPV$_{DNA+}$ tumors who are non-smokers have an even better clinical outcome, and that with each package of cigarettes their clinical outcome is worsens. Nevertheless, smokers with HPV$_{DNA-}$ OSCC have the poorest clinical outcome.
1.2.4 **Treatment for HNSSC and OSCC**

Early stage HNSCC is treated with either surgery or radiotherapy, often with good results. Unfortunately, these patients only represent one third of all cases. More advanced disease is usually treated with surgery, in combination with radiotherapy. In some cases chemotherapy with platinum based compounds is also used. Cetuximab (also Erbitux), a monoclonal antibody blocking EGFR, has also been used in combination with radiotherapy. This combination has in some cases been observed to yield better results than radiotherapy in combination with chemotherapy. In the case of metastasis or recurrent disease, different chemotherapy combinations and salvage surgery are often the only options. Cisplatin in combination with 5-fluorouracil (5-FU) is commonly used. However, even with all these treatment options, the prognosis is poor.\(^{134}\)

Due to this documented poor prognosis of HNSCC, treatment of all HNSCC including OSCC has been intensified the past decade with induction or concomittant chemotherapy, hyperfractionated radiotherapy, and in some cases Cetuximab. This treatment has lead to many more serious acute and chronic side effects, such as difficulties to eat, speak and breath and in many cases the patients have not been able to go back to an ordinary working life. In addition, treatment has been prolonged and the costs for society have increased.\(^{135}\)

It is also very doubtful if the majority of patients with HPV\(_{\text{DNA+}}\) OSCC need intensified treatment. However, since not all patients survive with standard radiotherapy, it is important to identify patients that will respond to therapy. Here, together with an HPV\(_{\text{DNA+}}\) or combined HPV\(_{\text{DNA+}}/\text{p16}\) positive tumour status, additional biomarkers maybe of use to better identify patients that will respond to therapy.
1.2.5. Biomarkers for prognosis in OSCC and HNSCC

Besides the presence of HPV, the roles of different biomarkers have been examined for their influence on clinical outcome in HNSCC and OSCC. Well known examples are such as the presence of p16, mutated p53 and other immunological markers such as MHC expression or tumor infiltrating lymphocytes.\textsuperscript{136-139}

In many studies HNSCC from different sites have been analyzed together and thus the specific location of the tumor not been taken into account. In addition, the HPV status of the tumors has often not been analyzed. It is now recognized that e.g. HPV-positive and HPV-negative OSCC should be considered as two different tumor entities and it is therefore crucial to define the HPV status of the tumors in studies of biomarkers for prognosis.\textsuperscript{130-132} The fact that the level of HPV infection differs between the different sites adds an extra dimension to this relation since e.g. HPV is very common in TSCC and BOTSCC, but almost absent in cancer of the hypopharynx.\textsuperscript{105, 115}

In this thesis we have focused on 3 different biomarkers in relation to prognosis CD4, CD8 and CD44.

CD8+ and CD4+ tumor infiltrating lymphocytes (TILs) are recognized as prognostic markers and have been studied in a number of cancer forms such as breast cancer, lung cancer and prostate cancer.\textsuperscript{140-143}

In these cases, numbers of CD4+ TILs have been both negatively and positively correlated to prognosis and numbers of CD8+ TILs have been positively correlated to prognosis.\textsuperscript{141, 144} An important function of CD8+ T-cells is to participate in the host defense during infection with viruses and intracellular bacteria by killing of infected cells. CD4+ T-cells on the other hand activate B-cells which in response will produce antibodies. They also produce cytokines which activate other parts of the immune system.\textsuperscript{145} Lymphocytes expressing either of these markers are therefore considered as important with regard to prognosis. Their exact role in cancer progression is however not fully elucidated.

The relation between CD44 and prognosis has also been studied in several cancer forms, e.g. lung cancer and breast cancer.\textsuperscript{146, 147} Most of the observations point to a negative correlation between CD44 and prognosis.\textsuperscript{148, 149} CD44 is involved in cell aggregation, proliferation, migration and angiogenesis. This cellular receptor has therefore been proposed to induce cellular proliferation of malignant cells. Upon binding of hyaluronic acid, crosslinking of tyrosine kinase receptors is mediated hence mediating proliferation.\textsuperscript{150} Therefore, it is reasonable to assume that absent/weak CD44 staining contributes to a better clinical outcome.

\textit{To find other biomarkers besides HPV has been one of the additional aims of this thesis.}
1.3 PREVENTION, SCREENING AND VACCINES

The most effective way to prevent oral and cervical HPV infection is most likely to avoid having sexual contact, and as mentioned above cervical cancer was rare in nuns. Also having few sexual partners may be associated with a lower oral and genital HPV prevalence.\(^{151}\)

It has however been discussed, whether HPV infection can be inhibited by distributing contraceptives which will protect against genital and oral infection, such as condoms and dental dams.\(^{152, 153}\) There is also a need for information and sexual education in order to educate the population on how to protect themselves against the negative factors associated with unprotected sex.\(^{154, 155}\)

Screening for cervical cancer, introduced by Papanicolaou\(^ {156}\), has been proven useful for early detection of pre-stages to cervical cancer and although not perfect its use has saved millions of lives in the countries where it has been introduced.\(^ {157}\) Today screening for HPV infection is suggested to be very useful e.g. for women above 35 years of age.\(^ {158, 159}\) For HPV-positive OSCC there are no implemented screening procedures and there is thus a need to evaluate the possibility to screen for HPV-positive OSCC.

*Therefore, to investigate the possibility to screen for HPV-positive OSCC has been an aim of this thesis. In addition, we studied whether different HPV16 variants were observed at different locations and if these variants were linked to clinical outcome.*

1.3.1 Prophylactic vaccines

Today, there are two prophylactic HPV vaccines commercially available, Gardasil® (GalaxoSmithKline, UK) and Cervarix® (Merck, USA). Both of these protect against infection with the two most common HR-HPV types, 16 and 18, covering approximately 70% of all HPV related cervical cancer. In addition, Gardasil® protects against LR-HPV6 and 11 which are the most common cause of condylomas.

The prophylactic vaccines are based on so-called virus like particles (VLPs), which are made up of the L1 capsid proteins that self-assemble into VLPs. The adjuvant used differs between the two vaccines. Cervarix® utilises aluminium hydroxide and is produced in insect cells, whereas Gardasil® contains amorphous aluminium hydroxyphosphate sulfae an is produced in yeast.\(^ {160}\)

When it comes to immune response and efficacy of the two vaccines, studies indicate very good protection from both vaccines with ~100% prevention of CIN III related to HPV16 and 18.\(^ {161}\) Studies investigating additional protective effects have been performed, indicating cross-protection against some other HR-HPV types such as HPV33, 31 and 52.\(^ {162}\) Cross protection is an important matter, since it may prevent aditional cervical cancers. More studies are however needed, since present data indicate varying levels of cross-protection (up to 40% in some cases) and also there is the possibility of shorter duration of cross-protection.\(^ {163}\)
The effector mechanism of both vaccines appear to be similar, since both evoke a clear antibody response. However, it is has been suggested that it is unlikely that CD4+ T-cell and CD8+ T-cell related effector mechanisms play a major role in prevention of infection. Cervarix® has been shown to evoke a more Th1 related response, while Gardasil® evokes a more Th2 related response. Moreover, although a strong CD8+ response is induced against L1 expressed in the upper layer of differentiated kertainocytes, it is unlikely that these lymphocytes come in contact with the HPV infected reproducing cells, and thereby erradicating them and curing the infection.164

Regarding the antibody response, it appears as if vaccination provides a much better initial response as compared to the antibody response caused by natural infection. A randomized placebo-control study from 2004 investigating bivalent vaccination, indicated that antibody titers were 100-times higher than those obtained after natural infection and 18 months after vaccination they remained up to 16 times higher and seemed to persist over time.165 Also, studies of memory B-cells from vaccinated women with no previous immunity towards HPV16 have been performed. Observations of HPV16-specific memory B-cells were made, however no tests were made of whether these cells gave rise to Abs in serum in this specific study.166

There are numerous studies of how efficient the two HPV vaccines are in protecting against HPV16 and HPV18 infection in the cervical tract.160-162 However, there are fewer studies on the ability of HPV vaccines to protect against oral HPV infection of which the article by Herrero et al was one of the first to show that HPV vaccination also reduces oral HPV infection.167

1.3.2 Therapeutic vaccines

There are no therapeutic HPV vaccines in use today, although many attempts have been made. Therapeutic vaccines generally aim to induce a strong Th1/cytotoxic T-cell response, which should be sufficient to clear a tumor driven by HPV antigens. Strategies tested so far include recombinant viral vectors and proteins, naked DNA, antigen pulsed dendritic cells and HLA-class I restricted peptide epitopes.

Even if one of these vaccination strategies would have induced a strong T-cell response other complications may lie ahead. HPV might still utilize some of its immunosuppressive features, which might be hard to overcome. Furthermore, mutations arising after the initial infection could potentially still drive tumor growth after virus eradication, making such a vaccine less useful.168

Two recent studies have reported 50% successful treatment of patients with HPV16 related high-grade vulvar lesions. The first vaccine was based on synthetic peptides of HPV16 E6 and E7 and the second one was based on recombinant HPV16E6E7L2 fusion protein. Both these vaccines managed to induce a strong CD4+ T-cell response and induction of CD8+ T-cells, both specific for HPV16. However these vaccines did not induce tumor regression in cervical cancer patients.168
2 AIMS

- Investigate the prevalence of HPV in the oral cavity of healthy youth, as well as in relation to cervical HPV prevalence

- Investigate oral HPV prevalence and cytology in patients with HPV\textsubscript{DNA+} TSCC and BOTSCC to examine whether it is possible to find useful screening methods

- Study whether there are different HPV16 variants in HPV-related tumors, compared to those in samples from healthy subjects

- Evaluate the influence of HPV-status and other biomarkers in TSCC and BOTSCC in response to therapy
3 MATERIAL AND METHODS

3.1 STUDY SUBJECTS

Several different cohorts were included in this thesis. Paper I included 490 healthy youth attending a youth clinic in central Stockholm, between 2009 and 2011. Paper II included 76 patients with suspected HNSCC visiting Karolinska University Hospital 2011 until 2013. In addition to the patients in Paper II, 37 dental patients attending the dental school at the Department of Dental Medicine at Karolinska Institutet were used as controls. Paper III had a total of 211 samples, including cervical samples from youth taken 2009-2011, cervical cancer biopsies and tonsillar cancer biopsies from patients diagnosed between 2000-2008 at Karolinska University Hospital. Papers IV and V included a cohort of almost 300 patients diagnosed with TSCC and BOTSCC between 2000 and 2007 at the Karolinska University Hospital.

3.2 PATIENT MATERIAL

Several different types of patient samples were used in the projects included in this thesis according to the list below.

Paper I

Mouthwash samples (50% water, 50% Listerine, totally 15ml) from 408 women and 82 men, 15-23 years of age, were included in the study. In addition, 180 women contributed with cervical samples collected with swabs (made out of nylon) in 5ml SurePath™. Of these women, 174 also contributed with oral samples (included in the total 408). Seven oral samples (all from women) were excluded due to lack of genomic DNA in the samples. All samples were collected at a youth clinic in central Stockholm between 2009 and 2011.

Paper II

Mouthwash samples, with the same formula as in Paper I, were collected from September of 2011 until June 2013 from 76 patients with suspected HNSCC. Tonsillar swabs from both the right and left side were also sampled by a nurse. Patients were recruited when they visited the department of ear-nose-throat surgery at Karolinska University Hospital (ENTKS), in order to undergo diagnostic endoscopy due to suspicion of HNSCC. In total, 29 TSCC, 18 BOTSCC and 19 other HNSCC/benign conditions were identified. In addition, 37 mouthwash samples and 24 pairs of tonsillar swabs were collected in 2011 from patients attending the dental school at the Department of Dental medicine at Karolinska Institutet.
Paper III

Three different sample types were used in this study which were obtained from patients either from the Karolinska University Hospital or a youth health center in Stockholm:
- 108 TSCC samples from patients diagnosed 2000-2007
- 52 Cervical cancer (CC) samples from patients diagnosed 2003-2008
- 51 Cervical samples (CS) from women attending a youth health center in Stockholm 2009-2011

Paper IV

In all, 275 formalin fixed paraffin embedded TSCC and BOTSCC biopsies, obtained from patients diagnosed 2000-2007, and earlier tested for HPVDNA and p16INK4a, were stained for both CD8+ and CD4+.

Paper V

The same initial cohort as in Papers III and IV was used in this project. This time 290 OSCC pretreatment biopsies were available for staining of CD44.

3.2.1 Ethical permissions

All studies included in this thesis were performed with permission from the Stockholm Regional Ethical Committee. List of permissions as follows:

- Paper I: 2008/870-31/4 and 2009/1147-31/2
- Paper II: 2009/1278-31/4 and 2010/1758-31/4
3.3 METHODS

3.3.1 DNA extraction

Samples and DNA extraction kits
DNA was extracted from several types of samples, such as e.g. mouthwash samples, tonsillar swab and cervical swab samples, and FFPE biopsies from different tumor samples. Three different kits were used for the DNA extraction:

- **Gentra Puregene Buccal Cell Kit** (Qiagen) Adapted to mouthwash samples
- **High pure PCR template preparation kit** (Roche) for oral and cervical swabs
- **High-pure RNA paraffin kit** (Roche) for FFPE material

3.3.1.1 The adapted version of the Gentra Puregene Buccal Cell Kit
The protocol for the Gentra Puregene Buccal Cell Kit was adapted according to sample type that was used, since this kit can be used for different sample types and of varying quality. The procedure was very much different compared to the other two kits, since filter tubes was not used in the protein extraction.

Samples containing a 15ml 50% Listerine/50% water mixture, which had been gargled 30sec by the patient, were stored at 4°C for a maximum of three days. The total sample was centrifuged and the pellet was washed in PBS. From this step, the pellet was resuspended and either extracted immediately, or as in most cases frozen down for extraction later on.

Upon DNA extraction, the pelleted cells were lysed in cell lysis solution, followed by protein lysis by Proteinase K. The lysed proteins were then pelleted upon centrifugation after protein precipitation solution was added. The supernatant containing DNA was then further processed by transfer into a mix of isopropanol and glycogen solution. At this stage the DNA “lumped together” and was easily spun down in a centrifuge with a built in cooling system. The pellet was then washed in 70% ethanol, air-dried and resuspended in DNA hydration solution, where after the DNA concentration was measured.

This kit was chosen for extraction of mouthwash samples, since it has been designed to remove enzymes and contaminants in an easy and effective way. Mouthwash samples contain proportionally large amounts of saliva, which in turn contains many degrading enzymes. It was therefore important to use a procedure, which was specifically designed for this sample type.
3.3.1.2 The High-pure RNA paraffin kit

The High-pure RNA paraffin kit was used to prepare DNA from FFPE-biopsies. The DNase step was removed from the protocol, hence a mix of both RNA and DNA was obtained. Fifteen micrometer thick sections (cut on a microtome at the core facility for histopathology at Cancer Center Karolinska) were deparaffinized with xylene and >96% EtOH. Then samples were mixed with tissue lysis buffer, 10% SDS, Proteinase K and left to incubate at 56°C overnight. The following day, the samples were treated with binding buffer and moved to a filter tube, treated with wash buffer I and II, followed by elution and measurement of DNA concentration.

As DNA and RNA in paraffin embedded material is often to some extent degraded, the choice of extraction method is of great importance. With this kit one can extract total RNA, but a mix of DNA/RNA is also achievable when removing the DNase step. By treatment with DNase or RNase pure DNA or RNA can be obtained, which gives the researcher the option of flexibility.

3.3.1.3 The High pure PCR template preparation kit

The procedure does not differ largely between the High pure PCR template preparation kit and the High-pure RNA paraffin kit. Samples were either buccal or cervical cells on swabs stored in SurePath™ solution at 4°C until preparation could begin, which was usually initiated within ≤ 3 days. The swabs were then vortexed in their 15ml storage tubes containing 5ml SurePath™, where after 1.5ml liquid was removed for further processing. The 1.5 ml samples were then mixed with 10mM TrisEDTA-buffer (pH8), tissue lysis buffer and Proteinase K and incubated at 55°C for 1hour. After the initial cell lysis, binding buffer was added followed by incubation at 70°C. Isopropanol was then added and the mix transferred to “High pure filter tubes”. The samples were then centrifuged and eluted with 70°C warm elution buffer and the DNA concentration was measured.

This kit is designed more for fresh material as compared to the The High-pure RNA paraffin kit. The option of using RNase to obtain pure DNA is available, instead of DNase as in the High-pure RNA paraffin kit above. As this procedure includes spin columns based on salt gradients, the need of organic solvents and DNA precipitation is eliminated, making it fast and easy to use.

3.3.2 Conventional and Multiplex PCR for HPV detection

3.3.2.1 Conventional PCR

In conventional PCR (also called standard PCR) only one target is amplified at the time. Although frequently used earlier this was not the main method for most projects included in this thesis. Multiplex PCR (described in the next section) replaced the conventional PCR in our lab to a large extent, as it offers an easier and more effective way of acquire the same information.
Conventional PCR had been used to some extent in the analysis of HPV DNA status on tumours included in Paper III-V. Samples in these cohorts were first tested by type specific PCR for HPV16E6. The type specific PCR was first performed for HPV16, as this is the most common type found in OSCC. If the initial analysis was negative, PCR with consensus primers followed. First primers GP5+/GP6+ were used, amplifying L1 of most known HPV-types. If this second test came out negative, the consensus primer pair CPI/CPIIG was used, amplifying E1 in case of L1 deletion. For samples still negative for HPV DNA, an additional PCR analysis for the cellular gene S14 was performed as a positive control for amplifiable cellular DNA. In Paper III, conventional PCR was used prior to sequencing of HPV16E6 in order to amplify the material of interest.

Example of a PCR protocol layout:  

<table>
<thead>
<tr>
<th></th>
<th>No of samples</th>
<th>PCR cycling program:</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR buffer(PE)</td>
<td>5 µl</td>
<td>95°C 4 min 1hold</td>
</tr>
<tr>
<td>dNTP (1.25mM/dNTP)</td>
<td>8 µl</td>
<td>95°C 30 sec 40cycles</td>
</tr>
<tr>
<td>MgCl2(25mM)</td>
<td>3 µl</td>
<td>49°C 30 sec 1hold</td>
</tr>
<tr>
<td>Primer.F (10pmol/µl)</td>
<td>2 µl</td>
<td>72°C 2 min</td>
</tr>
<tr>
<td>Primer.R (10pmol/µl)</td>
<td>2 µl</td>
<td>72°C 10 min 1hold</td>
</tr>
<tr>
<td>dH2O</td>
<td>24.8 µl</td>
<td>4°C</td>
</tr>
<tr>
<td>Taq pol gold (5U/µl)</td>
<td>0.2 µl</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>5 µl</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>45 µl</td>
<td></td>
</tr>
</tbody>
</table>

3.3.2.2 Multiplex PCR

Multiplex PCR was applied for all samples where HPV status was determined in Paper I and Paper II. This was done either for 24 HPV types (as in Paper I) or 27 HPV types (as in Paper II). The 24 HPV type PCR amplified the L1-region of HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 and 82. The additional types for the 27 HPV type set up were: HPV30, 67 and 69. These were not prioritized in the original set up since they are regarded as HPV types where there is limited or inadequate information regarding carcinogenesis.

HPV16E6 region was also included in the Multiplex 27 HPV type setup, since L1 might be deleted in some tumors it was of importance to investigate whether this was the case. HPV16 is the most common type associated with human cancer and variations of this type is therefore of great importance.

The cellular household β-globin gene was also included as a control for presence of genomic DNA. For the multiplex PCR reaction the Qiagen Multiplex PCR kit was used. The protocol was adapted depending on the type of sample with the total volume of the PCR reaction either 25µl (for FFPE samples and cervical swabs) or 50µl (in the case of mouth wash samples and oral swabs).
When the multiplex HPV PCR was set up the results were compared to those obtained by conventional PCR. Although the agreement was good between the assays we found that the multiplex PCR had a higher sensitivity and that less DNA was needed to obtain the same results.

Example of PCR layout for 50µl protocol:

<table>
<thead>
<tr>
<th>PCR</th>
<th>No of samples</th>
<th>1</th>
<th>µl/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer set 1 HPV (BSGP5+/6+ primer set)</td>
<td>2</td>
<td>0,5</td>
<td></td>
</tr>
<tr>
<td>Primer set 2 (b-globin primers)</td>
<td>0,12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P217-HPV16E6-1.F</td>
<td>0,076</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P234-HPV16E6-3.R</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2xQiagen Multiplex PCR Master Mix</td>
<td>12,304</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

One primer in each primer pair was biotinylated, so that a detection molecule could be added later on. Visualization was performed via a multiplex bead based assay, evaluated on a Magpix instrument supplied by Luminex (Figures12.). This instrument allows detection of as many as 50 specific targets in a small amount of sample, e.g. 5 ng tumor DNA or 5-10 µl of DNA preparation from cervical samples. Since the samples are analyzed in 96-well plates, this significantly lowers the workload and processing time of a standard PCR approach.

Magpix procedure:
The PCR products were mixed with a solution containing MagPlex® Microspheres (or beads), which are carboxylated polystyrene micro-particles, each with a unique fluorescent color. These beads are coupled to HPV specific probes enabling them to hybridize with single-stranded HPV amplicons upon denaturation at 95°C, followed by a 30 minute incubation at 41°C. Furthermore, a solution containing fluorescent streptavidin detection molecules was added, followed by further incubation at room temperature. The resulting DNA-bead-fluorecent streptavidin-complexes were visualized on a MagPix instrument, using two lasers (one red visualizing the beads and one green visualizing the detection molecule). Each bead has a unique fluorescent color allowing this detection. The amount of fluorescent streptavidin connected to each bead can also be measured and used as semiquantitative measure of the amount of the specific PCR product.
Figure 12. Schematic illustration of the bead-complex and illustration of the laser detection system used by the MagPix instrument. A red laser enables detection of the beads, whereas a green laser detects the detection molecule, e.g. fluorescent streptavidin.

The output is recorded in median fluorescent intensity (MFI) and is presented in an Excel based matrix displaying the MFI for each sample and individual bead type. In the current assay corresponding to different HPV types, all HPV types tested for at the top and all samples tested from top to bottom. A negative control is then used to determine the level of background noise. In addition, to avoid false positives, a factor of 15 was subtracted from the initial MFI output. The cut-off used for a positive sample was: Background*1.5 + 15. For sample types that generated mostly very weak results, there was no risk of false positives due to weak cross-reactions with other HPV types. In these cases a lower cut-off of was used: Background*1.5 + 8.

In addition, a β-globin level with an MFI >30 was considered as an indicator of sufficient DNA with a high enough quality for evaluation. This was especially important for HPV negative samples since HPV_{DNA+} samples did not need β-globin to be regarded as positive since the PCR obviously worked for the viral DNA.
Table 2. Primers included in the Luminex assay

**Forward**

<table>
<thead>
<tr>
<th>Name</th>
<th>sequence</th>
<th>gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP5+</td>
<td>5´-TTT GTT ACT GTG GTA GAT ACT AC-3´</td>
<td>L1</td>
</tr>
<tr>
<td>BSGP5+/-2</td>
<td>5´-TTT GTT ACT GTT GTI GAT ACT AC-3´</td>
<td>L1</td>
</tr>
<tr>
<td>BSGP5+/-3</td>
<td>5´-TTT GTT ACT GTT GTI GAT ACC AC-3´</td>
<td>L1</td>
</tr>
<tr>
<td>BSGP5+/-4</td>
<td>5´-TTT GTT ACT TGT GTI GAT ACT AC-3´</td>
<td>L1</td>
</tr>
<tr>
<td>BSGP5+/-5</td>
<td>5´-TTT TTA ACT GTT GTI GAT ACT AC-3´</td>
<td>L1</td>
</tr>
<tr>
<td>BSGP5+/-6</td>
<td>5´-TTT GTT ACT GTG GTA GAC ACT AC-3´</td>
<td>L1</td>
</tr>
<tr>
<td>BSGP5+/-7</td>
<td>5´-TTT GTT ACA GTI GTA GAC ACT AC-3´</td>
<td>L1</td>
</tr>
<tr>
<td>BSGP5+/-8</td>
<td>5´-TTT GTT ACA GTI GTA GAT ACC AC-3´</td>
<td>L1</td>
</tr>
<tr>
<td>BSGP5+/-9</td>
<td>5´-TTT GTT ACT GTG GTA GAT ACC AC-3´</td>
<td>L1</td>
</tr>
<tr>
<td>HPV16E6-1.F</td>
<td>5´- TCA AAA GCC ACT GTG TCC TGA -3´</td>
<td>HPV16 E6</td>
</tr>
<tr>
<td>HPV33E6.F</td>
<td>5´-TCG TTG GGC AGG GCG CTG TG-3´</td>
<td>HPV33 E6/E7</td>
</tr>
<tr>
<td>MS3.F</td>
<td>5´-AAT ATA TGT GTG CTT ATT TG-3´</td>
<td>β-globin¹</td>
</tr>
<tr>
<td>bglobin1170.F</td>
<td>5´-GTA CAC ATA TTG ACC AAA TCA GGG TAA-3´</td>
<td>β-globin¹</td>
</tr>
</tbody>
</table>

**Reverse (5´ Biotinylated)**

<table>
<thead>
<tr>
<th>Name</th>
<th>sequence</th>
<th>gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-GP6+</td>
<td>5´-GAA AAA TAA ACT GTA AAT CAT ATT C-3´</td>
<td>L1</td>
</tr>
<tr>
<td>Bio-GP6+-b</td>
<td>5´-GAA AAA TAA ATT GTA AAT CAT ACT C-3´</td>
<td>L1</td>
</tr>
<tr>
<td>Bio-GP6+-c</td>
<td>5´-GAA AAA TAA ATT GCA ATT CAT ATT C-3´</td>
<td>L1</td>
</tr>
<tr>
<td>HPV16E6-3.R</td>
<td>5´- GCT GGG TTT CTC TAC GTG TCC TG-3´</td>
<td>HPV16 E6</td>
</tr>
<tr>
<td>HPV33E6.R</td>
<td>5´- CTC GTG TCC TCT CAT GGC GTT-3´</td>
<td>HPV 33 E6/E7</td>
</tr>
<tr>
<td>Bio-MS10.R</td>
<td>5´-AGA TTA GGG AAA GTA TTA GA-3´</td>
<td>β-globin¹</td>
</tr>
<tr>
<td>bglobin1293.R</td>
<td>5´-GCC CTG AAA GAA AGA GAT TAG GGA AAG-3´</td>
<td>β-globin¹</td>
</tr>
</tbody>
</table>

¹In earlier studies MS3.F and Bio-MS10.R were used. These were later replaced by bglobin1170.F and bglobin1293.R with a higher annealing temperature and giving a shorter amplicon.
3.3.3 DNA-sequencing on an "Applied Biosystems 3730 DNA analyzer"

PCR-products were cleaned from excess primers and nucleotides by the use of ExoSAP-IT®, first heated to 37°C for 15 minutes followed by enzyme deactivation at 80°C for 15 minutes. The product is based on a combination of two enzymes Exonuclease I and Shrimp Alkaline Phosphatase (SAP). Exonuclease I works by removing the remaining single-stranded primers and left over single-stranded DNA, which has been produced during the PCR while SAP on the other hand, dephosphorylates residual dNTPs.171

The sequencing PCR was initiated after cleaning of the PCR-product. For this PCR automated dye-terminator sequencing was used. During the sequencing reaction, fluorescent dideoxynucleotides (ddNTPs) were incorporated in the DNA, terminating the reaction of the very same DNA strand, emitting light at different wavelengths.172, 173 The data was then recorded on an Applied Biosystem 3730 DNA analyzer.

Example of sequencing PCR layout and cycling program:

<table>
<thead>
<tr>
<th>HPV sequencing PCR</th>
<th>PCR-program</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>1</td>
</tr>
<tr>
<td>Big Dye mix</td>
<td>4 µl</td>
</tr>
<tr>
<td>2.5 Seq. dilution buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>Primer (1pmol/ul)</td>
<td>3 µl</td>
</tr>
<tr>
<td>Template</td>
<td>5 µl</td>
</tr>
<tr>
<td>H2O</td>
<td>6 µl</td>
</tr>
<tr>
<td>Total</td>
<td>20 µl</td>
</tr>
<tr>
<td></td>
<td>96°C 10sec</td>
</tr>
<tr>
<td></td>
<td>50°C 5sec</td>
</tr>
<tr>
<td></td>
<td>60°C 4min</td>
</tr>
<tr>
<td></td>
<td>25 cycles</td>
</tr>
</tbody>
</table>

3.3.4 Immunohistochemistry (IHC)

Immunohistochemistry was used for detection of CD8+ and CD4+ tumor infiltrating T-cells and for the expression of CD44 on the tumor cells with the following antibodies:

- CD44 clone: DF1485, Monoclonal mouse anti-human 1:1000 (Dako, Glostrup, Denmark)
- Anti-CD8 clone: 4B11, Monoclonal mouse antibody 1:40 (Novocastra)
- Anti-CD4 clone: 1F6, Monoclonal mouse antibody 1:40 (Novocastra)
- p16 Clone: JC8, Monoclonal mouse antibody 1:100 (Santa Cruz Biotechnology, Inc.)

Data from immunological staining of p16 was also used. Most staining and evaluation of this protein was performed in a routine setting at the Department of Clinical Pathology at the Karolinska University Hospital, Stockholm, Sweden, and if not, this was done by us.
Summary of standard protocol for IHC:

Slides were first de-paraffinized in xylene for 5 minutes, followed by lowering concentrations of ethanol á 5 minutes each (70, 95, 100%). Antigen retrieval was then performed by washing the slides, then placing the slides in citrate buffer in a water bath, which was brought to a boil in a microwave and then kept just below boiling point for totally 30 minutes. Following this, the slides were allowed to cool down and washed. Then blocking of unspecific binding with 1.5% horse serum was performed by adding this mixture to the slides for 30 minutes where after primary antibody was added (different dilutions depending on antibody) followed by incubation overnight.

The following day, the slides were washed and secondary antibody was added for 45 minutes. Slides were washed and an avidin-biotin complex (ABC) was used to biotinylate the secondary antibody. Horse radish peroxidase (HRP) was added in order to visualize the antibody staining in the following step. After 40 minutes of incubation, the slides were washed and developed with chromogen 3’ diaminobenzidine (DAB).

Finally, the slides were washed and placed in haematoxylin for 30 seconds in order to obtain counterstaining. Following this, the slides were washed for 5 minutes in running water, dehydrated in increasing concentrations of ethanol and xylene, mounted with coverslips and visualized under a light microscope.

3.3.4.1 Visual evaluation

Slides were evaluated by two independent individuals simultaneously, either counting the number of CD8+ and CD4+ tumor infiltrating lymphocytes (TILs) or analyzing the expression and intensity of the CD44 staining.

For CD4+ and CD8+ TILs, the evaluation was noted as: high, medium and low infiltration of TILs. The counted lymphocytes were those residing in in the tumor. These could however not be counted in areas close to veins and arteries since these cells might not have migrated into the tumor but instead just be passing by in the circulatory system. Staining of lymphocytes within the healthy tissue surrounding the tumor was used to determine the strength of positive staining.

With regard to CD44 expression, the tumors were evaluated by the percentage of CD44 positive cells and the intensity of staining. Staining intensity was evaluated as strong, weak or absent as compared to the surrounding normal tissue. Percentages were approximated based on the total amount of tumor present in each section and the percent of staining of the same.

The p16 staining performed in our lab was determined as positive or negative. A sample was regarded as positive when tumor tissue showed successful staining of >70% of the tumor.174
3.3.5 Statistical analysis

In all papers included in this thesis, statistical analysis was used to some extent. However the choice of statistical software varied.

Two definitions with regards to patient survival are of great importance:

**Overall survival** (OS) – follow up time for each patient was defined as time from diagnosis until death or in the case when patient was still alive after 3 years, they were considered censored at that time point.

**Disease free survival** (DFS) – follow up time was defined as lack of disease recurrence. Patients that died of their disease without prior recurrence were excluded from calculations, since they were considered not cured. Time of death from other causes (and without recurrence) was also a point for censoring.

**Statistical methods:**

In paper III and V, a **Chi2-test** was used to in order to compare unordered categorical variables, such as e.g. gender. This test is usually applied when the sample is large. However, in smaller samples, usually defined as a group < 5, a two-tailed **Fishers exact probability test** can be used instead, as in paper I, III and IV.

In paper V, an independent T-test was used to calculate differences in mean age between patient groups.

In paper III-V, survival was measured in years/days from the date of diagnosis until a defined event or to 3 years after diagnosis. Overall survival (OS) was defined as time from diagnosis until death of any cause. Disease-free survival (DFS) was defined as time from diagnosis until relapse in disease. Patients who died before a prior recurrence were censored at day 0 / death date. The **Kaplan-Meier** estimate was used to estimate OS and DFS, followed by a **log-rank** test for differences in survival between groups, e.g patients with HPV$_{DNA+}$ versus HPV$_{DNA-}$ tumors.

Unadjusted and adjusted Hazard ratios (HR) were calculated with a **Cox regression** for data in Paper IV. This method investigates the effect of several variables upon the time a specified event happens, e.g. death by disease. This function will provide better estimates of survival probability and cumulative hazard ratios as compared to a Kaplan-Meier calculation, if the assumptions are met. This method was further used in Paper V, creating a univariate and a multivariate **Cox regression model**.

The **Wilcoxon-Mann-Whitney** test (WMW) is generally a test for the comparison of two independent random samples. This was used in Paper IV to investigate differences in continuous and ordered categorical variables, e.g. counts of CD8+ cells.
Paper I also included calculations for HPV16 concordance between genital and oral samples. This was performed by calculating the **Kappa statistic**. This value gives a numerical rating of the degree of chance by which two values agree. A perfect agreement will give you a kappa value of 1 and an agreement occurring by chance gives a value of 0.

In addition, in Paper II box-plot calculations were performed with R-statistical software (version 3.0.1), graphically investigating the differences in MFI in-between patients and healthy youth.

Other software used were GraphPad Software for Paper III, SAS software (ver. 9.3, SAS Institute Inc., Cary, NC, USA) in Paper IV and SPSS (IBM SPSS Statistics, version 20, Stockholm, Sweden) in Paper V.
4 RESULTS AND DISCUSSION

4.1 PAPER I.

Prevalence of oral human papillomavirus infection among youth, Sweden

**Aim:** To investigate the prevalence of oral HPV infection at base line in young men and women attending a youth clinic in Stockholm 2009-2011 before the introduction of public HPV vaccination in Sweden.

**Study subjects, Methods and Results**

In all, 408 women and 82 men aged 15-23 years of age were included in the study contributing with mouthwash samples, and 180 women also donated cervical swabs. All samples were tested for 24 different mucosal HPV-types by the use of a Luminex-based multiplex assay. Seven oral samples and one cervical sample were excluded due to insufficient material.

In total, 9.3% of the oral samples were HPV-positive with 9.2% among women and 9.8% among men. Cervical HPV infection was observed in 74.1% of the cases, and 64.9% of these were caused by HR-HPV types in the 174 women donating a cervical sample as well as an oral sample. HPV16 was the most common HR HPV-type, present in 2.9% of all oral samples and 37.9% of all cervical samples. Infections with more than one HPV type was frequent in the cervix and found in the majority of the cases, while only 16% of the HPV positive mouthwash samples presented more than one HPV type. Of note, in general the MFI values were much higher for HPV in cervical samples as compared to samples from the oral cavity.

Oral and cervical samples from 174 women were paired and among the 24 women with an oral HPV infection, 22 of them also had a cervical HPV infection. Furthermore, there was HPV type concordance for 20 of 22 (90.9%) cases between the two sites.

Notable was also that oral HPV infection was more common in women with cervical HPV infection (17.1%) compared to those without cervical HPV infection (4.4%).

Table 3. HPV prevalence in mouthwash samples and cervical samples from youth, Stockholm, Sweden. Adapted from Du et al. 2012

<table>
<thead>
<tr>
<th>HPV-positive categories</th>
<th>Oral</th>
<th>Cervical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any HPV</td>
<td>45 (9.3)</td>
<td>129 (74.1)</td>
</tr>
<tr>
<td>High risk-HPV</td>
<td>35 (7.2)</td>
<td>113 (64.9)</td>
</tr>
<tr>
<td>HPV16</td>
<td>14 (2.9)</td>
<td>66 (37.9)</td>
</tr>
<tr>
<td>HPV18</td>
<td>1 (0.2)</td>
<td>35 (14.49)</td>
</tr>
<tr>
<td>Total no. samples</td>
<td>483</td>
<td>174</td>
</tr>
</tbody>
</table>
Discussion

The main goal of this project was to investigate oral HPV prevalence of healthy in youth, before public HPV vaccination.

With this aim in mind it could be argued that the cohort was biased. Firstly, the cohort may not be representative of average youth of the same age. Since the youth visiting the clinic were there to consult for birth control and sexually transmitted diseases (SDTs) and likely to have had more sex partners and hence be at greater risk of infection with any SDT. In addition, the size of the cohort included in the study was relatively limited since ~4000 women and 800 men visit the clinic each year, but only 408 women and 82 men enrolled. Consequently, the actual test group had the potential to be larger and sampling could have been more evenly distributed during the time period whilst the study was ongoing and between women and men. In addition, the sampling frequency varied over time depending on the workload of the nurses at the clinic, and whether there were many patients visiting or if many nurses were on sick leave. In all these cases the nurses did not have time to ask visitors to contribute. The gender distribution is explainable since the female’s visitors outnumber the male visitors 5 times, a ratio which is reflected in our cohort where almost 500 women as compared to only 82 men contributed with samples. Individuals whom are sexually active provide a good basis for sample collection and a youth clinic represents a very good place for collecting these types of samples. The probably higher oral HPV prevalence among this group is an advantage to establish the relative frequency of different HPV types and the possible effect of HPV vaccination on oral prevalence.

The oral HPV prevalence observed, i.e. 9.3% in the whole cohort (9.2% in women and 9.8% in men), was somewhat higher but relatively comparable with other studies both in the US and other Western countries REF.175 Later studies of more population-based cohorts have shown a lower oral prevalence, both among youth and other age groups. The fact that 70% of the cervical infections were caused by HR-HPV types was similar to a previous study at this youth clinic and expected.81 Notably, HPV MFI signals, giving a semi-quantitative assessment of the copy number of HPV DNA in the samples, were lower in oral as compared to cervical samples and multiple cervical infections were more frequent as compared to multiple infections in the oral cavity. The lower HPV MFI signals obtained in oral samples as compared to oral samples could for partly be due to that in the oral cavity, the samples are diluted with saliva, which is produced in the magnitude of 0.5-1.5 liters/day.

The 174 paired cervical and oral samples disclosed some interesting data. First of all, oral infection was more common in women with cervical infection (17.1%) compared to those without cervical infection (4.4%). Furthermore, of the 22 women with simultaneous oral and cervical infection there was a 90.9% HPV-type concordance, suggesting that they may have been acquired from the same partner.

Conclusion: Oral HPV prevalence was 9.3% in youth and equal among women and men at a youth clinic in central Stockholm, before public HPV vaccination and oral infection was more common in women with, as compared to women without cervical HPV infection.
4.2 PAPER II.

*Human papillomavirus prevalence is high in oral samples of patients with tonsillar and base of tongue cancer*

**Aim:** To study if patients with HPV\textsubscript{DNA+} TSCC and BOTSCC could be distinguished from patients with other HNSCC by HPV\textsubscript{DNA} testing mouthwash samples and/or tonsillar swabs.

**Patients, Methods and Results**

The study included 76 patients with suspected HNSCC, admitted to the Karolinska University Hospital for a diagnostic endoscopy. Definite diagnosis of HPV in the tumor biopsies was collected from patient journals, and compared to results from the non-invasive sampling by mouthwash or tonsillar swabs. As HNSCC free controls, 37 dental patients from the Dental School at Karolinska Institutet were included.

Of the 76 patients with suspected HNSCC, 29 patients were diagnosed with TSCC and 18 patients with BOTSCC, with 76% and 89% of the respective cases being HPV\textsubscript{DNA+} according to the patient records. From patients with HPV\textsubscript{DNA+} TSCC, 82% presented oral samples with HPV type concordance with the tumor biopsy, while the corresponding figure for HPV\textsubscript{DNA+} BOTSCC was 50%. Of the remaining 29 patients, 19 presented HNSCC other than TSCC and BOTSCC and 10 had benign conditions such as tonsillitis or fungal infections, and of all these patients 4/29 (14%) had HPV\textsubscript{DNA+} oral samples.

The majority 27/32 (84%) of the HPV\textsubscript{DNA+} oral samples were thus obtained from 26 patients with HPV type concordant TSCC or BOTSCC and in one case a patient with an unknown primary of the head and neck. HPV16 dominated in TSCC and BOTSCC and the MFI values were high, indicating a high viral load. More specifically, HPV16 MFI values in mouthwash samples from patients with TSCC and BOTSCC were generally higher compared to those in mouthwash samples from healthy youth. Figure 13 shows a boxplot for HPV16 MFI values of oral samples from patients with TSCC and BOTSCC as compared to those obtained from youth aged 15-23 years in Paper I (with an average MFI of around 250 and 20 respectively). The dental patients had an oral HPV\textsubscript{DNA+} prevalence of 8%. This was similar to the prevalence reported among youth in Paper I (9.3%), but somewhat lower than observed in patients with HNSCC other than TSCC and BOTSCC and other conditions (14%).

In addition, tonsillar swab samples from 60 patients: 22 TSCC, 15 BOTSCC and 25 from HNSCC other than TSCC and BOTSCC and benign conditions were sent and successfully evaluated with cytology at the Regina Elena National Tumor Institute and San Galliciano Institute of Dermatology. Clearly malignant cells were found in 7% (4/60) of the samples, whereas 33% (20/60) were classified as ASCUS (Atypical Squamous Cells of Undetermined Significance). Of those with clearly malignant cytology 3/4 were contributed by patients with TSCC, while the final sample was from a patient presenting with cancer of the mobile tongue. The 20 ASCUS samples were evenly distributed among the diagnoses, 7 TSCC and 6 BOTSC and 7 other HNSCC and benign conditions. Notable was that 50% of HPV\textsubscript{DNA+} TSCC that could be evaluated had ASCUS or clearly malignant samples.
Discussion

The purpose of this project was to investigate to which extent patients with HPV\textsubscript{DNA}\textsuperscript{+} TSCC and BOTSCC had HPV\textsubscript{DNA}\textsuperscript{+} oral samples and could be distinguished from other HNSCC patients. Testing for HPV\textsubscript{DNA} in oral samples from 76 patients undergoing an endoscopic diagnostic biopsy for the suspicion of a HNSCC, it was shown that 89\% of HPV\textsubscript{DNA}\textsuperscript{+} oral samples were from patients with HPV\textsubscript{DNA}\textsuperscript{+} TSCC and BOTSCC or an HPV\textsubscript{DNA}\textsuperscript{+} unknown primary. Furthermore, if limiting the analysis to HPV16\textsubscript{DNA}\textsuperscript{+} samples with MFI signal >200, 100\% of the HPV16\textsubscript{DNA}\textsuperscript{+} oral samples were derived from TSCC, BOTSCC or an unknown primary of the head and neck. Thus an HPV\textsubscript{DNA}\textsuperscript{+} oral sample, especially HPV16\textsubscript{DNA}\textsuperscript{+} with a relatively high viral load, is very likely belonging to a patient with an HPV\textsubscript{DNA}\textsuperscript{+} TSCC or BOTSCC.

Nevertheless, there were HPV\textsubscript{DNA}\textsuperscript{-} oral samples from patients with HPV\textsubscript{DNA}\textsuperscript{+} tumors. The overall the reliability of the method was higher for HPV\textsubscript{DNA}\textsuperscript{+} TSCC as compared to HPV\textsubscript{DNA}\textsuperscript{+} BOTSCC, where 82\% as compared to 50\% respectively of the oral samples were HPV\textsubscript{DNA}\textsuperscript{+}. The most plausible reason for this higher sensitivity is that when sampling tonsillar swabs and mouthwash samples one most likely enrich for cells from the tonsils rather than from the base of tongue, which is further down in the oral cavity. It is possible that by taking swabs from the base of tongue one could increase the number of HPV\textsubscript{DNA}\textsuperscript{+} oral samples obtained for patients with BOTSCC. It was also noted that oral samples from HPV33\textsubscript{DNA}\textsuperscript{+} TSCC and BOTSCC were more often HPV33\textsubscript{DNA}\textsuperscript{-}, probably due to a lower sensitivity for HPV33 in the assay.

In addition, 14\% of patients with other HNSCC or benign conditions and 8\% of the dental patients also had HPV\textsubscript{DNA}\textsuperscript{+} oral samples, indicating that not only patients with TSCC or BOTSCC may have positive oral samples. This was not unexpected since oral HPV\textsubscript{DNA} prevalence of both these cohorts was fairly similar to that obtained among youth (9.3\%) at the youth clinic in Paper I. However, none of these patients had HPV16 with MFI >200.
The above finding prompted us to compare MFI values from patients with HPV\textsubscript{DNA+} TSCC and BOTSCC with those obtained among youth at the youth clinic. Here we found an obvious difference since HPV16 MFI values in youth had a median MFI of 20 as compared to a median MFI of 250 among patients with HPV\textsubscript{DNA+} TSCC and BOTSCC.

Finally, cytopathological data from tonsillar swabs collected from 60 patients with sufficient material displayed varying results. Clearly malignant cytology was not obtained for most patients this way. Material was inadequate or not available for 7 of the 22 patients with HPV\textsubscript{DNA+} TSCC and among the remaining 15 samples, seven showed malignant cytology or ASCUS (47%). The sensitivity for BOTSCC was much weaker, which was not unexpected, and very likely the sensitivity would have been improved if a swab had been taken from the base of the tongue instead.

**Conclusions**

- HPV\textsubscript{DNA+} oral samples were mainly derived from patients with HPV\textsubscript{DNA+} TSCC and BOTSCC, with somewhat lower sensitivity in the latter group.

- When comparing HPV16 MFI values in mouthwashes from patients with HPV16\textsubscript{DNA+} TSCC and BOTSCC and youth there were considerable differences i.e. with average MFI median values of 200 and 20 respectively.

- Based on our findings in Papers I and II, we conclude that individuals with HPV16\textsubscript{DNA+} oral samples with relatively high HPV viral loads should be checked for the possible presence of an HPV\textsubscript{DNA+} TSCC or BOTSCC.
4.3 PAPER III.

*Human papillomavirus (HPV) 16 E6 variants in tonsillar cancer in comparison to those in cervical cancer in Stockholm, Sweden*

**Aim:** To investigate HPV16 E6 variants in tonsillar cancer (TSCC) as compared to those in cervical cancer (CC) and cervical samples (CS) of healthy young women.

**Patients, Material, Methods and Results**

FFPE material of HPV-positive TSCC was randomly selected from a cohort of 290 patients with TSCC diagnosed in Stockholm between 2000 and 2007 (see Figure 14). CC samples were selected from a cohort of patients diagnosed between 2003 and 2008. Totally 55 TSCC and 52 CC samples were chosen for the initial comparison, later an additional 53 TSCC from the original TSCC cohort were included for validation. Furthermore, 51 CS samples were chosen from a previous study.81

![Flow chart of patients with TSCC included in the study](image)

Figure 14. Flow chart of patients with TSCC included in the study

Sequencing for HPV16 E6 was first performed for an initial group of samples (55 TSCC, 52 CC and 51 CS). The most common variant found in all groups was the L83V, which was observed in 45% of the TSCC, 31% of the CC and 29% of the CS. Notably, the otherwise rare mutation R10G was rather common (22%) in the TSCC samples, rare in the CS samples (4%) and completely lacking in the CC samples. The differences were found to be significant between both TSCC and CC (p=0.0003) as well as for TSCC compared to CS (p=0.009).
When analyzing the frequency of these two variants in 53 additional HPV16 positive TSCC samples a similar result was achieved with 17% R10G and 34% L83V positive samples. No significant difference was observed when summarizing the two groups of TSCC, and the total frequencies obtained for R10G and L83V were 19% and 40%, respectively.

Notably, the majority of samples containing R10G also harbored L83V. Other mutations were detected in all sample groups, but these were however uncommon in comparison and of these the two most common ones (Q14H and H78Y) were present in only 4% of the CC and CS and 7% of the TSCC cases.

When considering HPV16E6 variants with different geographic origins the European prototype was found in 38% of the TSCC, 65% of the CC and 59% in the CS. If one includes samples harboring the European variant with minor differences (such as R10G and L83), the percentage increased to >90% for all sample types. Other prototypes of HPV16E6 such as African-1 and 2, East Asian, Asian American and North American were rare and only observed in a few samples.

No significant differences were observed among patients with TSCC with regard to TNM-status, stage or 3-year disease-free survival (DFS) when comparing the ones with or without the R10G and L83V.

Discussion

In this study, the aim was to investigate whether there were any differences between HPV16E6 variants observed in TSCC, CC and CS, indicating that there could be differences in the affinity or ability of different variants to survive at different locations.

The common L83V found in 45% of the TSCC, 31% of the CC and 29% of the CS implied that there were no major differences with regard to the location of this variant, although it has often been suggested to be more prone to cause cervical cancer. Furthermore, the similar frequencies of L83V in CC and CS samples, argues against this hypothesis. Notable was that the R10G variant, which is in general very rare, was present in 19% of the TSCC samples, while it was only present in 4% of the CS samples and completely absent in the CC samples. This suggests that this variant may be more able to adapt itself in the oral cavity or alternatively that it is more prone to cause cancer in this region.

A limitation of this study was that no oral samples from healthy individuals were included. This was mainly due to that we only had 12 HPV16 positive oral samples from the study on youth from the youth clinic and that these samples had a very low HPV signal making sequencing difficult. However, in future studies it may be valuable to embark on such a task. It would namely be useful to know, whether this variant is more common in the oral cavity and therefore present in TSCC or if it is more carcinogenic as such as compared to other HPV16 variants.

Presence of different variants was also correlated to clinical outcome in TSCC, but no significant differences were observed. However, with the limited number of patients and the high survival in this patient group such differences are difficult to detect.
Conclusions

- L83V was fairly common in TSCC, CC and CS and was distributed equally between the latter two and not enriched for in CC

- The rare mutant R10G was found in TSCC but not in CC, while in CS it was found in 4% of the cases. From the presently obtained data it cannot be determined whether R10G was selected against in CC, instead further studies are needed.

- Finally, none of the above variants showed any significant impact on patient clinical outcome
4.4 PAPER IV.

*CD8*⁺ and *CD4*⁺ tumor infiltrating lymphocytes in relation to human papillomavirus status and clinical outcome in tonsillar and base of tongue squamous cell carcinoma

**Aim:** To estimate the numbers of *CD8*⁺ and *CD4*⁺ tumor infiltrating lymphocytes (TILs) in TSCC and BOTSCC and in combination with HPV-status in relation to clinical outcome.

**Patients, Materials, Methods and Results**

For this study, 280 FFPE tumor biopsies were obtained from a cohort of patients diagnosed with TSCC and BOTSCC and treated with curative intent between 2000 and 2007. All samples had been previously tested for presence of HPV DNA and expression of p16INK4a (p16). Immunohistochemistry was used to stain *CD8*⁺ and *CD4*⁺ TILs, and the number of TILs was estimated by two independent researchers.

In all, 79% of the tumors were HPV DNA positive (HPVDNA⁺) with HPV16 being present in 94% of the cases. The remaining 6% were positive for either HPV33, 35, 56, 58 or 59. Patients with HPVDNA⁺ TSCC were in general younger than patients with HPV DNA negative (HPVDNA⁻) tumors and had a lower T-stage. Patients with HPVDNA⁺ BOTSCC had a higher nodal stage and clinical stage compared to those with HPVDNA⁻ tumors. No significant difference in gender prevalence depending on HPV DNA status was observed. p16 overexpression was observed in 82% of the HPVDNA⁺ tumors and in 8% of the HPVDNA⁻ ones (p<0.001) with a slightly higher prevalence among patients with TSCC.

TILs can be regarded as important for the immune response and the number of *CD8*⁺ TILs has been previously shown to be important for clinical outcome in other tumors and therefore of interest to study also here.⁷⁷

*CD8*⁺ TILs could be evaluated in 275/280 (98%) of the tumors and *CD4*⁺ TILs in 268/280 (96%) of the cases. Significantly higher numbers of *CD8*⁺ TILs as well as *CD4*⁺ TILs cells were found in the HPVDNA⁺ group of tumors.

Notably, high *CD8*⁺ TIL, but not *CD4*⁺ TIL counts were found to be a favorable prognostic factor among patients with HPVDNA⁺ as well as HPVDNA⁻/p16-positive tumors. This was especially obvious after dividing the patients into 4 quartiles, observing 3-year overall survival (OS) and disease free survival (DFS), which was significantly better for all but the group with the lowest *CD8*⁺ TIL counts (Figure 15). For patients with HPVDNA⁻ tumors there was a similar tendency, however statistical significance was obtained only for OS and for the quartile with the highest *CD8*⁺ TIL counts.

In contrast, high *CD4*⁺ TIL counts were not found to affect clinical outcome in patients with HPVDNA⁺ or HPVDNA⁻/p16 positive tumors. Nevertheless, a tendency of better survival for patients with HPVDNA⁻ and HPVDNA⁻/p16 negative tumors was observed.
Figure 15. Kaplan-Meier curves displaying OS and DFS for HPV\textsubscript{DNA+} TSCC (A and B respectively) and HPV\textsubscript{DNA+}/p16-positive TSCC (C and D respectively), with data stratified for CD8\textsuperscript{+} TIL counts divided into 4 quartiles.

Discussion
The aim of this study (and Paper V as follows below) was to investigate the use of additional biomarkers in combination with HPV-status in TSCC and BOTSCC to better predict clinical outcome and to potentially better select patients with HPV\textsubscript{DNA+} tumors for randomized trials with less aggressive therapy than that given today.

Both Paper IV and V make use of the same cohort i.e. patients diagnosed with TSCC and BOTSCC 2000-2007 at the Karolinska University Hospital. In this study 280 biopsies were available as compared to 290 biopsies in Paper V. The reason for the discrepancy is due to that it was not always possible to obtain enough material in order to stain for all markers.

In this study 79\% of the TSCC and BOTSCC samples were HPV DNA positive, with dominance of HPV16. In all 82\% of HPV\textsubscript{DNA+} cases were also p16 positive, while only 8\% of the HPV\textsubscript{DNA-} tumors were p16 positive. Since being HPV\textsubscript{DNA+}/p16 positive is regarded as almost equivalent to being HPV E6/E7 mRNA positive, i.e. the golden standard for a HPV driven tumor, clinical outcome was investigated for this group too.
In general, HPV\textsubscript{DNA-} tumors displayed higher counts of CD8\textsuperscript{+} TILs than HPV\textsubscript{DNA+} tumors. Therefore, when making comparisons of the importance of CD8\textsuperscript{+} TILs, the two groups were evaluated separately. This division may explain why the three highest quartiles for HPV\textsubscript{DNA+} and HPV\textsubscript{DNA+}/p16 positive groups all were correlated to a better clinical outcome. Accordingly, in order for patients with HPV\textsubscript{DNA-} cancer patient group to benefit from high CD8\textsuperscript{+} TIL counts, they had to belong to the quartile with the highest count of infiltrating cells.

Infiltration with CD4\textsuperscript{+} TILs could not be linked to clinical outcome or survival for patients with HPV\textsubscript{DNA+} tumors, although there was a tendency of better survival for patients with HPV\textsubscript{DNA-} and HPV\textsubscript{DNA-}/p16-negative tumors with high CD4\textsuperscript{+} TIL counts. This issue would be interesting to unravel and followed up in later studies in larger cohorts of HPV\textsubscript{DNA-} cancer if possible. On the other hand it is unlikely that treatment will be de-escalated for this patient group within the near future and therefore such a study would not be a first priority.

Conclusions

- Patients with HPV\textsubscript{DNA-} and HPV\textsubscript{DNA-}/p16 positive TSCC and BOTSCC which belong to the three highest quartiles of CD8\textsuperscript{+} TIL counts have a significantly better clinical outcome than those belonging to the lowest quartile

- For patients with HPV\textsubscript{DNA-} cancer only those with the highest quartile of CD8\textsuperscript{+} TIL counts have an increased survival

- CD8\textsuperscript{+} TIL counts have the potential to be used as a biomarker to predict clinical outcome in HPV\textsubscript{DNA+} and HPV\textsubscript{DNA+}/p16 positive TSCC and BOTSCC
4.5 PAPER V.
Absent/weak CD44 intensity and positive human papillomavirus (HPV) status in oropharyngeal squamous cell carcinoma indicates a very high survival

Aim: To investigate whether CD44 could be used as a potential biomarker to identify patients with HPV DNA positive (HPV\textsubscript{DNA+}) or HPV\textsubscript{DNA+}/p16 positive TSCC and BOTSCC with a better clinical outcome.

Patients, Material and Results
In all, 290 patients with TSCC or BOTSCC diagnosed between 2000 and 2007 and with available tumor biopsies were included in the study. Totally, 78\% of the tumors were HPV DNA positive with HPV16 accounting for 94\% of the cases. Out of the HPV\textsubscript{DNA+} cases 90\% were also p16 positive, the same was true for 8\% of the HPV\textsubscript{DNA-} cases. Clinical outcome was evaluated for patients with HPV\textsubscript{DNA+} tumors and for those with HPV\textsubscript{DNA+}/p16-positive tumors, where the latter are regarded as more likely to have an active HPV infection. Clinical outcome was also evaluated for patients with HPV\textsubscript{DNA-/p16} negative tumors. Patients with HPV\textsubscript{DNA+} or HPV\textsubscript{DNA+/p16} positive tumors were younger at diagnosis, had smaller tumors, and higher level of nodal disease and stage than those with HPV\textsubscript{DNA-} cancer. No significant differences were observed with regard to gender, presence of metastasis or tumor localization.

A sample was regarded as CD44-positive when >25\% of the cells displayed CD44 staining. Among the 290 tumors included, 92\% expressed CD44 with a majority displaying strong intensity staining (53\%), while 26\% had intermediate staining and 21\% showed weak staining. Patients with CD44-positive tumors showed higher differentiation and had larger tumors to a significantly higher degree than those considered as CD44-negative. However, CD44-positivity alone was not correlated to clinical outcome irrespective of HPV status and was therefore not pursued further.

The impact of CD44 intensity staining was therefore investigated. This was done by dividing the samples into two groups, absent/weak- and medium/strong-CD44 intensity staining. It was then shown that HPV\textsubscript{DNA+} samples more often expressed medium/strong intensity staining, as compared to HPV\textsubscript{DNA-} tumors (91\% vs. 65\%, p<0.001).

Furthermore, when considering CD44-intensity staining in relation to DFS and OS, it was noted that an absent/weak CD44 intensity staining lead to a better 3-year DFS and OS for all tumors, irrespective of HPV status as compared to those expressing medium/strong CD44 intensity staining. A univariate analysis and multivariate analysis was performed (Table 3) showing that patients with HPV\textsubscript{DNA+} tumors had a better clinical outcome than those with HPV\textsubscript{DNA-} tumors. Furthermore, patients with tumors with absent/weak CD44 intensity staining had a significantly better DFS and OS than those with medium/strong CD44 intensity staining.

Investigating HPV\textsubscript{DNA+} and HPV\textsubscript{DNA-} (Table 3, Figure 16) and HPV\textsubscript{DNA+/p16} positive and HPV\textsubscript{DNA-/p16} negative tumors (Figure 17) performing a Kaplan-Meier analysis also yielded useful information.
Patients with HPVDNA+ tumors and absent/weak CD44 staining were again found to have a better 3-year DFS and 3-year OS than those with tumors with medium/strong CD44 intensity staining, while the difference was not significant for the smaller HPVDNA- group (Table 3).

For patients with HPVDNA+/p16 positive with absent/weak CD44 intensity staining there was a trend of a better 3-year DFS and 3-year OS (Figure 16), as compared to those with tumors with medium strong CD44 intensity staining, but the values did not reach statistical significance. For patients with HPVDNA-/p16 negative, no additional significant increase in 3-year DFS or 3-year OS was recorded in the group with absent/weak CD44 intensity staining as compared to that with medium/strong intensity staining (Table 3).

Figure 16. Disease free survival in patients with HPVDNA+/p16 positive tumors in relation to CD44 intensity staining of their tumors.
**Table 3A.** Univariate and multivariate analyses in HPVDNA+ patients of CD44 intensity, p16 expression and clinical parameters (data not shown) for 3-year disease free and overall survival.

<table>
<thead>
<tr>
<th></th>
<th>Disease free survival</th>
<th>Overall survival</th>
<th>Disease free survival</th>
<th>Overall survival</th>
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<td><strong>CD44 intensity</strong></td>
<td></td>
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<tr>
<td>absent/weak</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
<td>(ref)</td>
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<tr>
<td>medium/strong</td>
<td>3.4 1.0 - 11.6</td>
<td>0.047</td>
<td>3.9 1.4 - 10.9</td>
<td>0.011</td>
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<tr>
<td><strong>p16 expression</strong></td>
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<td></td>
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<tr>
<td>absent</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td>present</td>
<td>0.67 0.16 - 2.9</td>
<td>0.59</td>
<td>0.16 0.076 - 0.32</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval

* All listed factors are included in the regression model.

**Table 3B.** Univariate and multivariate analyses in HPVDNA- patients of CD44 intensity, p16 expression and clinical parameters (data not shown) for 3-year disease free and overall survival.

<table>
<thead>
<tr>
<th></th>
<th>Disease free survival</th>
<th>Overall survival</th>
<th>Disease free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD44 intensity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>absent/weak</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td>medium/strong</td>
<td>1.7 0.22 - 12.6</td>
<td>0.62</td>
<td>1.8 0.43 - 7.4</td>
<td>0.43</td>
</tr>
<tr>
<td><strong>p16 expression</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>absent</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
<td>1 (ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td>present</td>
<td>0.85 0.19 - 3.7</td>
<td>0.83</td>
<td>0.84 0.30 - 2.4</td>
<td>0.75</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval

**Table 4.** Univariate and multivariate analyses for 3-year OS and DFS for patients with HPVDNA+ and HPVDNA- tumors. Parameters included: CD44 intensity and p16 expression. Clinical parameters were also included in the multivariate analysis (age, sex, stage and tumor site), data not shown.
Discussion
The aim of this study was to investigate the use of CD44 as a biomarker in combination with HPV-status in TSCC and BOTSCC to better predict clinical outcome and to evaluate the future use of personalized therapy.

HPV positive status was examined as HPVDNA+, or HPVDNA+/p16 positive, where the latter is regarded to be close to the golden standard and indicative of an active HPV infection. HPVDNA- status was examined as HPVDNA- or HPVDNA-/p16 negative.

Irrespective of HPV status, most tumors stained positive for CD44. However, CD44 positivity as such did not have an impact on clinical outcome. However, when examining differences in CD44 intensity staining, a new picture emerged. It was observed that patients with tumors with absent/weak intensity staining had a better clinical outcome than those with medium/strong CD44 intensity staining. This marker was hence further pursued.

When investigating CD44 intensity staining further, it was found that patients with HPVDNA+ tumors with absent/weak CD44 intensity staining had a significantly better 3-year DFS and 3-year OS than patients with medium/strong CD44 intensity staining. For patients with HPVDNA- tumors the same trend was observed, however the number of patients was limited and statistical significance was not obtained. For patients with HPVDNA-/p16 positive tumors with absent/weak CD44 intensity staining there was a trend for a better 3-year DFS and 3-year OS, but this trend did not reach statistical significance. Thus there was a difference with regard to the use of CD44 intensity staining as compared to CD8+ TILs in this group of patients suggesting that CD8 TIL counts may be a better biomarker for clinical outcome in patients with HPVDNA-/p16 positive tumors (see Paper IV).

Moreover, when comparing the HPVDNA+ group to the HPVDNA-/p16 positive group one can observe a cumulative survival increase both for DFS and OS in the group with CD44 medium/strong intensity staining in the latter group.

This was most likely due to exclusion of all patients with the HPVDNA-/p16 negative samples, since these patients do worse as compared to patients with HPVDNA-/p16 positive samples. We hypothesize that this in part could explain why a statistical significant difference was not observed between patients with absent/weak vs. medium/strong HPVDNA+/p16 positive tumors.

Conclusions
- Among patients with TSCC and BOTSCC, and patients with HPVDNA+ TSCC and BOTSCC absent/weak as compared to medium/strong CD44 intensity staining indicated a significantly better 3-year DFS and OS
- In patients with HPVDNA+/p16 positive and HPVDNA-, TSCC and BOTSCC absent/weak as compared to medium/strong CD44 intensity indicated a trend for better 3-year DFS and OS. For this purpose the impact of CD44 intensity as a predictive marker needs to be investigated further
5 CONCLUSIONS

- Oral HPV infection was relatively common among youth attending a youth clinic in Stockholm 2009-2011, before public HPV vaccination as compared to other studied populations in Europe and the U.S. (Paper I)

- Oral HPV infection was more common in women with genital infection and there was HPV type concordance between the oral and cervical sites (Paper I)

- When testing patients with suspected HNSCC, HPV_{DNA+} oral samples were mainly derived from patients with HPV_{DNA+} TSCC and BOTSCC (Paper II)

- The relative HPV viral load (Median Fluorescent Intensity) was significantly higher in mouthwash samples of patients with HPV_{DNA+} TSCC and BOTSCC as compared to similar samples from healthy youth. This makes non-invasive mouthwash or other oral samples potentially a basis for developing routine testing for HPV_{DNA+} TSCC and BOTSCC (Paper I and II)

- The HPV16E6 L83V variant was common in TSCC, CC and CS, while the rare HPV16E6 R10G variant was present in a proportion of TSCC, but absent in CC and only sporadic in CS samples (Paper III)

- Neither the HPV16E6 common L83V nor the rare R10G variants had any significant impact on clinical outcome. However, it is possible that the R10G variant is more adapted to infecting the tonsils or that is more prone to cause TSCC than the European prototype. Hence further investigation of this variant can be of interest (Paper III)

- Both high CD8+ TIL infiltration and absent/weak CD44 intensity staining appeared to be promising predictive markers for patients with HPV_{DNA+} TSCC and BOTSCC. However for patients with HPV_{DNA+}/p16 positive TSCC and BOTSCC only CD8+ TILs retained statistical significance, indicating that this marker was the better one of the two (Paper IV and V)
6 FUTURE PERSPECTIVES

In Paper I we monitored oral and cervical HPV-prevalence in young sexually active individuals prior to public vaccination and found high HPV-prevalence for both the cervical and oral tracts. However, studies that we have performed on HPV-prevalence need to be followed up since public HPV vaccination has been initiated from 2012. Our hope is that infection with HPV16 and 18 will decrease (together with HPV6, HPV11 and other types affected by cross-protection), both in the anogenital area and in the oral cavity. This decrease is likely to contribute to a decreased number of cervical cancers but also to a decrease in HPV related cancers of the head and neck among both men and women.

The insight that patients with HPV\textsubscript{DNA}+ TSCC and BOTSCC have higher viral load in the oral cavity, as compared to HPV\textsubscript{DNA}+ healthy youth, gives new possibilities for future research. Furthermore, the finding that patient with HPV\textsubscript{DNA}+ HNSCC usually doesn’t show oral positivity adds to this potential. One should aim to develop an easy-to-use non-invasive test, which can be utilized by health care staff to give a specific and quick answer. This test should provide information whether there is an ongoing HPV infection and whether the patient should go through further examination as to rule out HPV\textsubscript{DNA}+ TSCC or BOTSCC from the diagnosis.

Studies on different variants of HPV16E6 are of importance, especially since we found that the R10G variant is more common in TSCC as compared to in CC and in CS. This was an observation in patients from the Stockholm County and whether this is the case also in samples from other areas needs to be further investigated. In addition, since no significant impact on clinical outcome was observed, possibly due to the limited number of samples examined, further investigation in larger cohorts could be considered.

Considering the results from Paper IV and V, further investigation of biomarkers is of great importance. If one had a strong panel of biomarkers, with high accuracy identifying patients with HPV\textsubscript{DNA}+ TSCC and BOTSCC with better clinical outcome, then personalized medicine would not be science fiction. One could then investigate the potential benefit of deescalated treatment. However, it is crucial to validate the beneficial effects of high CD8\textsuperscript{+} TILs and absent/weak CD44 intensity staining in larger groups of patients, before attempting to use them to perform randomized trials with de-escalated therapy.
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Max my sunshine, Let’s have a wonderful future!

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